

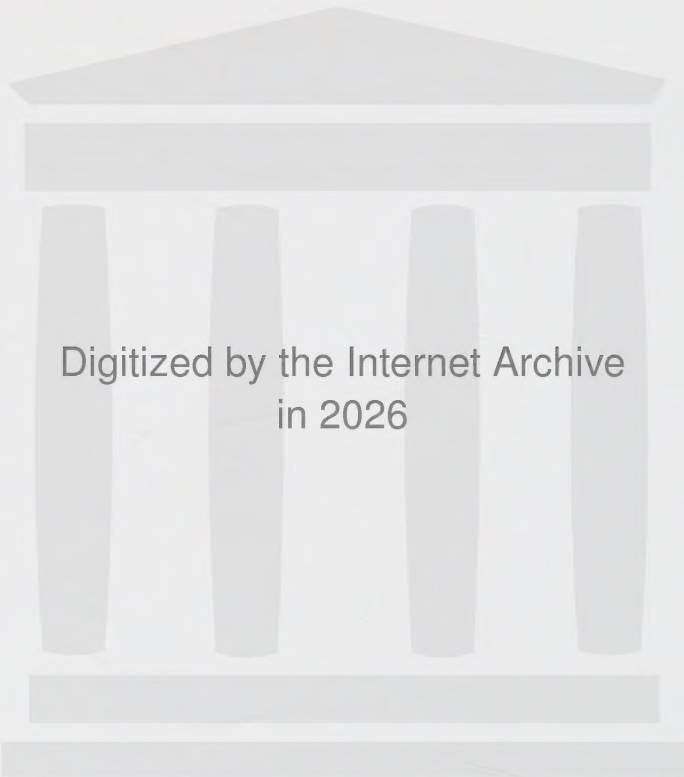
Studies on the Structure and Expression of β Globin Genes

INAUGURAL DISSERTATION

zur Erlangung der Philosophischen Doktorwürde
vorgelegt der Philosophischen Fakultät II
der Universität Zürich

von
ANTON SCHAMBOECK
von Basel

Begutachtet von
Herrn Prof. Dr. C. WEISSMANN



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Meinen Eltern

Für die Möglichkeit experimentelle Erfahrungen zu sammeln und einen Einblick in ein aktuelles Gebiet der genetischen Forschung zu erhalten bin ich Herrn Prof. Weissmann zu Dank verpflichtet. Sein umfassendes Wissen, seine fundierten Kenntnisse und die zielstrebige Art zur Lösung wissenschaftlicher Fragestellungen waren eine wichtige Grundlage für die vorliegende Arbeit.

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P. Curtis, W. Boll und J. Ecsödi haben mir in grosszügiger Weise präparative Erzeugnisse wie Enzyme, Plasmide und Kulturen von bester Qualität zur Verfügung gestellt.

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SUMMARY

In order to study the structure and function of "single copy" eukaryotic genes, we undertook to isolate the rabbit beta globin gene. In collaboration with J. van den BERG and A. van OUYEN, newly developed techniques of recombinant DNA technology were applied and the chromosomal beta globin gene of the rabbit was cloned in a bacterial plasmid.

In the first part of this work rabbit chromosomal DNA was isolated from freshly prepared liver. High molecular weight DNA was cleaved with the restriction endonuclease Kpn I to generate a fragment of 4800 - 5200 base pairs containing the entire beta globin gene including the 5' and 3' flanking regions (JEFFREYS & FLAVELL 1977). This fragment was purified about 20 to 50 fold by large scale preparative agarose gel electrophoresis. After elongation with dA-residues and annealing to dT-elongated pCRI plasmid DNA, the hybrid molecules were introduced into *E. coli* X1776. More than 50 000 individual colonies were screened by colony hybridization, using as probe a 333 base pair Hae III restriction fragment containing part of the coding sequence of a cloned complementary DNA copy of rabbit beta globin mRNA (p β G, MANIATIS et al. 1976).

Analysis of positive colonies resulted in the identification of one clone, which contained a DNA insert with the same restriction map as deduced for rabbit chromosomal DNA by the SOUTHERN blotting procedure (JEFFREYS & FLAVELL 1977).

The sequence coding for beta globin is not contiguous in the genomic DNA. Two interruptions called introns or intervening sequences, one of 125 base pairs close to the 5' end and one of 650 base pairs between the Bam and EcoRI sites about in the middle of the gene, were demonstrated in cloned DNA by restriction mapping (Van den BERG et al. 1978). The complete nucleotide sequence of the rabbit beta globin gene including 5' and 3' flanking regions was determined by Van OUYEN et al. 1979.

In the second part of this work the molecular structure of a mouse beta globin 15s precursor RNA was established. 10s-beta-globin mRNA results from processing of a 15s precursor RNA (CURTIS et al. 1977). It seemed likely that the 15s precursor is an exact copy of the genomic DNA and that 2 fragments (introns) of 105 base pairs and of 650 base pairs are excised from the precursor RNA. The most convincing data for the structure of 15s precursor RNA would be sequence analysis. RNA sequencing would however be very difficult on the minute quantities of 15s RNA which can be isolated. Since DNA sequencing is much easier to carry out, we used the 15s beta-globin RNA as a template to generate a double-stranded DNA copy by reverse transcriptase and cloned it by the usual techniques. To identify 15s globin cDNA clones, colonies were first screened by hybridization to globin mRNA; in a second round of hybridization, the positive clones were screened with a probe specific for the large intron. The positive clones were analysed by restriction site mapping and the DNA sequence of one of the clones (called M15s- β G-1), which contained beta-globin specific DNA including the small and large intron was determined. Two clones representing possible splicing intermediates of beta major globin (M15s- β G-3) and of beta minor globin (M15s- β G-6) were identified.

INTRODUCTION:

WHAT IS A GENE:

The definition of a gene has changed with the development of biology. Originally "gene" was an operational concept and defined a heritable unit which gave rise to a discrete phenotypic trait. Genes for different traits were found to recombine during sexual propagation, giving rise to new phenotypes. The gene appeared to behave as the unit of recombination and at the same time as a functional unit. Genes were then found to be changed by mutations and were also defined as the unit of mutation. It later became apparent, on finer analysis, that the unit of recombination and of mutation was actually the nucleotide, and thus did not coincide with a unit of function.

The concept of the gene was refocussed with the "one gene - one protein" hypothesis of Beadle, Tatum and Horowitz (HOROWITZ & LEUPOLD 1951), which with the substitution of "polypeptide" or "structural RNA" for "protein", appears as an appealing basis for a definition for a gene.

An operational definition which is applicable (with limitations) in prokaryotic systems, was introduced by BENZER 1961, who coined the new term "cistron". It is very useful in fast propagating phage systems: Cells are coinfectd with two independent mutant phages creating a trans configuration; each mutation lies on a different strand. If the two mutations affect different cistrons, complementation of the two defective genomes and phage propagation is observed. The cis configuration is obtained by a mixed infection with wild type and double mutant. A cistron is the segment on a chromosome that behaves as a functional unit in the cis-trans test. A cistron thus corresponds to the DNA segment giving rise to one polypeptide. In higher eukaryotic, slowly propagating systems, the operational definition cistron has not become very popular.

At the same time JACOB & MONOD 1961 defined the operon, a coordinately expressed gene cluster in prokaryotes (the lac operon). It consists of the regulatory regions, promotor and operator at the transcriptional initiation site, the structural genes and the termination signal. A polycistronic mRNA is formed and translated into the functional proteins. This raises the question as to whether the promotor and operator (cis-active sequences) are to be included in the definition of a gene (as unit of function); if so, then regulatory regions controlling a coding sequence may be physically remote from the latter.

The functional unit of eukaryotic genetic material has an even more complex structure. The simple "one gene one protein" hypothesis is certainly not sufficient to describe the present day knowledge of gene systems. The concept of "gene", although useful in superficial discussions, is in practice best replaced by the concepts "transcriptional unit" and "translational unit". The transcriptional unit is a segment on the DNA, which is transcribed into RNA beginning at the initiation site up to the termination site. The translational unit is a composite of the segments on the DNA which ultimately result in a polypeptide chain. Both structural RNAs and functional proteins frequently result by cleavage of the primary transcription or translation products. For the DNA segments represented in the mature product I suggest the term "maturation unit".

Some examples of eukaryotic gene systems are briefly described.

(I) A number of genes produce functional RNAs which are not translated into proteins, as the ribosomal RNAs and the transfer RNAs. The transcriptional unit may give rise directly to a functional RNA, but frequently the primary transcript becomes functional only after further processing. In this step the ends can become shorter ("processing") or internal sequences are removed ("splicing") and nucleotides are modified, resulting in the mature rRNA or tRNA, corresponding to the maturation unit.

(II) The mRNA for most eukaryotic proteins is derived from a longer precursor (transcriptional unit) by a fast processing

step in which internal sequences, called introns or intervening sequences are removed and the adjacent regions (exons) spliced together, giving rise to mature mRNA (mRNA maturation unit). A part of this work deals with the structural relationship between the mouse beta globin 15s precursor RNA and its chromosomal counterpart.

(III) In the case of polypeptide hormones a longer precursor protein (polyprotein, corresponding to the translational unit) is cleaved by specific proteases into functional fragments (peptide maturation units).

(IV) In SV40 the same transcript (transcriptional unit) is spliced in two alternative ways, leading to two different mRNAs (mRNA maturation units) producing the two functional polypeptides small t and large T antigen (translational units). In ϕ X174 phage a case of two overlapping transcriptional units is known, both are translated into proteins in different reading frames. These are examples of "overlapping genes".

In the case of the beta globin gene the transcriptional unit represents the transcribed beta globin precursor sequences including initiation and termination signals. The primary transcript is processed to mature mRNA (mRNA maturation unit), and translated into the beta globin polypeptide (translational unit). The functional hemoglobin consists of two alpha and two beta polypeptides and one heme molecule.

THE PURPOSE OF THE PROJECT:

The rabbit beta-globin gene is an ideal system to study the structure and function of a "single copy" eukaryotic gene. The amino acid sequences of beta-globin chains from many different species have been described (DAYHOFF et al. 1972). The complete nucleotide sequence of rabbit beta-globin mRNA was determined (MANIATIS et al. 1976, BARALLE 1977). The beta-globin protein is relatively small, containing 147 amino acids encoded by a mRNA of 582 nucleotides. It was therefore thought, that there would be a good chance to obtain the entire gene with its flanking regions in one restriction fragment and to get access to the functional significant parts.

An interesting biological system to study globin gene expression and its induction is the erythroleukemic Friend-cell system. these cells are blocked in an early stage of erythropoetic development and can be artificially induced to undergo further differentiation and to produce globin by dimethylsulfoxide treatment.

In a first stage the primary structure of the chromosomal rabbit beta-globin gene was determined and its nucleic acid sequence compared with the known amino acid and RNA sequences and with the beta-globin sequences of other species. The regions preceding the cap and following the poly-A addition site were examined for striking arrangements.

In a second step, in vitro and in vivo systems were explored, which allow the expression of the beta-globin gene to be studied. The formation and stability of RNA and proteins can be tested by specific assays (BERK & SHARP 1977, MULLIGAN et al. 1979, HAMER et al. 1979). Techniques exist or were developed to introduce the cloned gene into bacteria (Van den BERG et al. 1978), yeast (BEGGS et al. 1980) or eukaryotic cells (MULLIGAN et al. 1979, WIGLER et al. 1979, MANTEI et al. 1979).

Currently modified genes or gene segments are used instead of the original gene, and the effect of these modifications will be monitored in order to determine the functional significance of a particular gene segment.

GENE ORGANIZATION IN EUKARYOTES

BRITTEN & KOHNE 1968 undertook an extensive study of eukaryotic chromosomal DNA by hybridization- and renaturation kinetics. Total cellular DNA was denatured, annealed under standardized conditions and the fraction of double-stranded DNA was determined as a function of time and concentration. It was concluded from the renaturation kinetics that there are three main components of DNA in eukaryotic genomes: unique DNA sequences present in one to ten copies per haploid genome (including closely related sequences), intermediate repetitive sequences with a repetition frequency of more than 10^6 . The highly repetitive DNA appears not to be transcribed, since the RNA sequences present in eukaryotic cells correspond only to the unique and the intermediate repetitive sequences (FLAMM et al. 1969).

Examples of repetitive genes are the ribosomal and transfer RNA genes, the histone genes and the immunoglobulin kappa chain genes; and for so-called single genes the rabbit beta-globin-, the ovalbumin- and the chicken insulin genes. For all of these genes additional closely related sequences have been reported which are sometimes not expressed but cross-hybridize with a gene specific probe (LACY et al. 1979).

The total number of actively transcribed genes in a eukaryotic cell was estimated to be not more than 50 000 (OHTA & KIMURA 1971). The amount of DNA needed to code for this number of polypeptides was estimated and compared to the actual DNA content per cell. It was found that a 10 to 100 fold excess of DNA is present (OHNO 1971, JUDD et al. 1972).

Some possible explanations why eukaryotic cells contain an excess of DNA may be given by observations described recently. RNA extracted from nuclei after a short pulse of radioactivity shows a characteristic class of RNA, called heterogenous nuclear RNA (hnRNA). The size of this RNA is greater than that of the bulk of mRNA, and after a short chase a large portion of the hnRNA can no longer be detected in the nucleus or anywhere else! hnRNA might consist of various precursor

RNAs and indicate that the majority of the genes contain introns. In the case of the mouse beta-globin gene more than 50% of the chromosomal DNA is not included in the globin coding sequences. Intron DNA may account for a portion of the excess DNA in eukaryotic genomes.

To date little is known about the mechanism of eukaryotic gene regulation at the molecular level. However, regulatory sequences may well account for some of the excess DNA found in eukaryotic cells.

A third phenomenon has been observed during development and differentiation. During a specific stage in differentiation a unique pattern of polypeptides and corresponding mRNAs was observed. The same RNAs could not be detected in later stages of differentiation. This indicates that certain genes are activated only for a short period during differentiation (SIM et al. 1979).

The phenomenon of somatic rearrangement during differentiation is well documented for immunoglobulin genes by the structural comparison of embryonic and adult chromosomal immunoglobulin sequences (BRACK et al. 1978). Mechanisms must be postulated which control these DNA transfers. Some DNA sequences postulated to participate in these transfers may well account for another portion of excess DNA.

Another class of DNA, the satellite DNA consists mainly of repetitive sequences and its function in the cell is not clear at present.

It is still possible that a significant portion of eukaryotic DNA is not involved in physiological processes but represents a silent pool of genetic potential, or contributes to chromosomal organization as a structural part only.

GENE ISOLATION PROCEDURES:

Increasing interest in the molecular and biochemical behaviour of gene expression has led to the development of methods to produce specific gene segments in large amounts and to their isolation in pure form. One of the first successful attempts in a prokaryotic system was described by SHAPIRO et al. 1969 for the isolation of pure lac operon DNA. The method is not of general applicability but demonstrates the basic idea of using a lambda-phage vector to propagate a specific DNA segment. These authors used two related but not identical phages, which transduce a part of the lac operon in different orientations at high frequency. Following heteroduplex formation and degradation of the single-stranded phage arms with the single-strand specific nuclease S_1 of *Aspergillus oryzae*, pure lac sequences were obtained. It is remarkable that this experiment was done by the methods of classical genetics without the use of restriction endonucleases or ligase.

The genome of a eukaryotic cell is 10^3 to 10^4 times more complex than of a prokaryotic cell and gene purification is a more serious problem.

Physical methods: Much effort has been put into DNA purification procedures and will be discussed briefly.

Highly repetitive histone genes from sea urchin have been purified with great efficiency by buoyant density gradient centrifugation (BIRNSTIEL et al. 1975, 1977).

Transfer RNA genes were significantly enriched by repeated density gradient centrifugation in the presence of silver chloride (CLARKSON et al. 1967, 1978).

The purification of single-stranded DNA was initially favoured and methods were adapted from viral systems (COFFIN et al. 1974) for application to the rabbit globin-system by HOFSTETTER (thesis 1976). The RNA of rabbit reticulocytes contains 60 - 70% globin mRNA, which was converted into complementary DNA by reverse transcriptase and elongated with

50 to 100 deoxy-C-residues by terminal transferase. This dC-elongated globin complementary DNA was hybridized to chromosomal DNA which had been cleaved by a restriction enzyme and totally denatured. By passing the whole mixture over poly-I-Sephadex (COFFIN et al. 1974) globin DNA hybrids were selectively retained, and after elution with formamide a fraction of single-stranded globin plus strand DNA was obtained, 500 to 2000-fold enriched with a yield of 5 to 10%. Similarly a procedure for the enrichment of minus strand was developed by FLAVELL (personal communication) using mercurated mRNA for hybridization to cellular DNA, which was eluted from sulfhydryl-Sepharose with beta-mercaptoethanol (DALE et al. 1975). A 5000-fold enriched preparation of minus strands was obtained. When plus and minus strand preparations were reannealed, joined to plasmid vectors and transfected into bacteria no globin containing colonies could be detected and so far no successful report has appeared, which followed this technique. Considerable purification of ovalbumin-specific chromosomal DNA was obtained by ANDERSON & SCHIMKE 1976. They coupled ovalbumin cDNA covalently to cellulose following the procedure described by NOYES & STARK 1975.

A very similar theoretical approach was proposed by YENIKOPOLOW et al. 1976. Sheared cellular DNA was treated with exonuclease III which acts on 3' ends and produces extended single-stranded regions. Gene sequences were exposed in this way to a single-stranded probe and enriched by the methods described above. However, no successful cloning experiment has been described following this model.

THOMAS et al. 1976 described conditions under which an RNA-DNA hybrid is more stable than the corresponding DNA-DNA hybrid. This allows a partial displacement of one DNA strand by the RNA and gives rise to a so called R-loop structure. TONEGAWA et al. 1977 used immunoglobulin mRNA to form R-loops with cellular DNA. Following successive CsCl density gradient

centrifugation he obtained an enriched DNA fraction which was used to clone immunoglobulin genes in a lambda vector.

Two methods are now generally used for the partial purification of double-stranded DNA. The first one is preparative electrophoresis of restriction fragments on agarose gels, which separates DNA according to size. For this purpose, automated equipment has been engineered which allows continuous fractionation and elution of DNA from the gel (POLSKY et al. 1978). The second method separates DNA according to size and AT-content and allows the rapid fractionation of relatively large amounts of DNA: reverse phase high pressure liquid chromatography or RPC-5. The matrix consists of powdered polyethylene which is coated with a quaternary ammonium base (LANDY et al. 1976, HARDIES & WELLS 1976, ESHAGPOUR & CROTHERS 1978, LARSON et al. 1979). Both methods can be applied in succession resulting in purifications up to 500-fold.

Molecular cloning: The most powerful technology has been the development of gene cloning procedures. Cloning uses the concept of in vitro recombination and ligation of DNA fragments. In general, cellular DNA fragments are ligated to a vector DNA and used to transform recipient host cells. The recipient cells are then screened by a specific "gene assay". CLARKE & CARBON 1975 presented a simple system using the restriction endonuclease EcoRI. EcoRI cleavage of cellular DNA produces staggered ends. The plasmid vector DNA was cleaved by the same enzyme and hybrid molecules were formed using T₄-DNA-ligase. An antibiotic resistance marker located on the vector DNA allowed selection of transformed cells after the transfection of the hybrid-molecules into an E. coli host. The plasmid DNAs in the single colonies which were obtained represented pure cloned cellular DNA fragments and religated vector sequences. One of the first successful cloning experiment of a eukaryotic repetitive gene was reported by MORROW et al. 1974 for ribosomal DNA of *Xenopus laevis*.

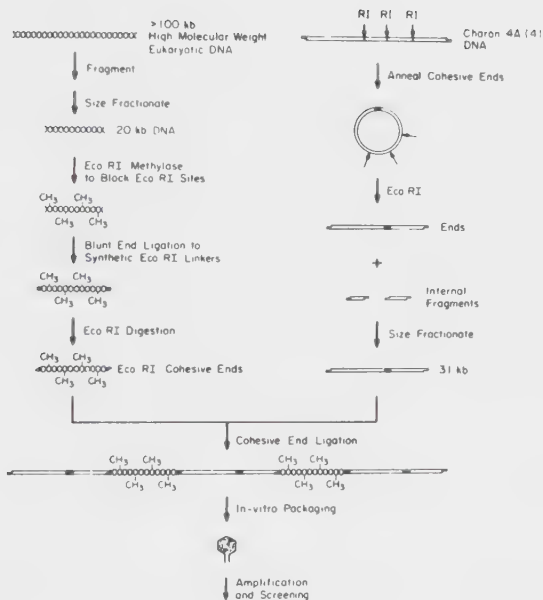
An alternative procedure for cloning was proposed by JACKSON et al. 1972 and LOBBAN & KAISER 1973. They used the enzyme

terminal transferase which preferentially elongates 3' overhanging ends of DNA fragments. Fragmented cellular DNA was elongated by deoxy-A residues and annealed to deoxy-T elongated vector DNA. The circular hybrid molecules were used to transform bacterial cells. The frequency of clones containing just vector sequences was less than one in 1000. The method is of very general applicability.

DNA inserts joined by AT-tailing can be excised from the vector DNA as shown by HOFSTETTER et al. 1976. Hybrid-DNA was partially denatured in formamide at elevated temperature and the low melting AT-stretches were preferentially attacked by the single-strand specific nuclease S_1 .

At present the most frequently applied cloning strategy is the so called "shotgun experiment". Total cellular DNA is cleaved at random either by shearing or by partial restriction cleavage and a sufficient number of clones are produced to represent the total cellular genome. The larger the integrated DNA fragments, the smaller the number of clones required to cover the entire genome and the higher the probability that the desired gene is completely present in a single fragment.

A collection of clones representing the whole genome of an organism is called a gene bank. Gene banks have been established for a number of organisms including man, namely: *E. coli* (CLARKE & CARBON 1976), yeast (KRAMER et al. 1976), *drosophila* (GLOVER et al. 1977, SCHEDL et al. 1978), chicken (DODGSON et al. 1979), rabbit and man (MANIATIS et al. 1978). For low complexity organisms as *E. coli*, yeast and *drosophila* 10 000 to 50 000 single clones are stored systematically in microtiter dishes. More complex eukaryotic organisms would require $5 \cdot 10^5$ to 10^6 single clones and can no longer be stored singly in microtiter dishes. The approach to establish a gene bank of a higher eukaryote, as proposed by MANIATIS et al. 1978 is outlined in Scheme 1. Hybrid phage DNA molecules, containing the eukaryotic DNA segment are packaged into lambda heads in vitro, and bacterial cells containing a temperature sensitive lambda-repressor are infected with the particles.



Scheme 1:

Strategy used to construct libraries of random eukaryotic DNA fragments by MANIATIS et al. 1978.

High molecular weight DNA was fractionated by partial cleavages with the restriction endonucleases AluI or BspI in order to obtain blunt ends for the ligation with synthetic EcoRI linkers.

The gene bank can be amplified as a bacterial culture and can either be stored in aliquots after induction of the phage by temperature shock in the form of a lysate, or can readily be spread in a plaque assay. A possibly serious problem is the counterselection and disappearance of variants with slow growth characteristics. At present this problem has not yet been extensively studied.

Vector systems: Much effort was put into the construction of plasmid vectors: pCRI by CARBON 1976, pMB9 by BOLIVAR et al. 1977 and the best characterised plasmid pBR322, sequenced by SUTCLIFFE 1978.

A vector system which is used with great success is phage lambda. A number of phages have been constructed which have proven to be very useful in gene cloning. MURRAY 1975 removed the middle non-essential part of the phage and purified the right and left arms preparatively. Cellular DNA fragments containing EcoRI ends of about 5 kb in length have been ligated to the lambda arms and propagated in bacteria. BLATTNER et al. 1977 constructed 16 different "charon" phages which take fragments from 1 to 10 kb in length containing EcoRI, HindIII or SstI restriction sites.

A similar phage (λ gtWES λ B) carrying EcoRI fragments of one to 12 kb was constructed by LEDER et al. 1977 and TIEMEIER et al. 1976. Its practical value was demonstrated by the cloning of the mouse beta major globin gene by LEDER et al. 1978 and the immunoglobulin genes by TONEGAWA et al. 1977, a breakthrough in cloning technology.

A considerable advantage of the lambda-cloning system is the possibility of packaging ligated hybrid DNA into phage particles by an in vitro procedure (HOHN 1974, 1977, STERNBERG et al. 1977). A few hundred micrograms of host DNA are sufficient to generate a phage collection representing a whole eukaryotic genome. The frequency of transformed cells after phage infection is 100 to 1000-fold more efficient than after transfection with phage or plasmid DNA.

Cosmids are vectors which contain the origin of replication of a bacterial plasmid and the cohesive ends of phage lambda. Hybrid molecules with up to 40 kb long inserts can be prepared and packaged into phage particles. After infection the DNA molecules are amplified like plasmid DNA (COLLINS & BRUNING 1978).

Gene-assay: Of invaluable help in gene purification methods and hybridization assays is the availability of large amounts of a highly specific probe. Messenger RNA is in most cases not pure enough. The viral enzyme reverse transcriptase is able to convert mRNAs into almost full length double-stranded DNAs which can easily be integrated into plasmid vectors and cloned in bacterial cells. ROUGEON et al. 1975 and MANIATIS et al. 1976 realized the construction of rabbit globin cDNA plasmids. The products are called genes by some authors, but it should be noted that these products are artificial, nonphysiologic compounds. Nevertheless a number of cDNA plasmids derived from eukaryotic mRNAs have been successfully expressed in bacterial cells.

THE GLOBIN GENES:

The globin genes direct the synthesis of various species of globin polypeptides which, in conjunction with heme, form hemoglobin. The most abundant hemoglobin in adult species is composed of 2 alpha and 2 beta globin polypeptides and one heme molecule. The main function of hemoglobins is the transport of oxygen from the lungs to all the other tissues and of transport of carbon dioxide from the tissues back to the lungs. Hemoglobin is found in only one type of cell, the erythrocyte and its immediate precursors.

Amino acid sequences of globin polypeptides have been determined for many different species and are collected and evaluated in DAYHOFFS Atlas of Protein Sequences. (DAYHOFF et al. 1972)

Only few data are reported on the sequences of globin mRNA. Partial sequences of the rabbit alpha and beta globin mRNA were presented by PROUDFOOT & BROWNLEE 1976, and for human globin mRNAs by FORGET et al. 1975, CHANG et al. 1977 and BARALLE 1977.

The complete mRNA sequences were determined by DNA-sequencing of cloned cDNA plasmids. MANIATIS et al. 1976 presented the complete sequence of rabbit beta globin mRNA, HEINDELL et al. 1978 the rabbit alpha globin mRNA. The mouse alpha and beta major cDNAs were sequenced by MANTEI 1978 (personal communication). Cloned cDNA plasmids of human globin mRNAs have been prepared and characterised by LITTLE et al. 1978. A fetal sequence was reported by COGGINS et al. 1978.

The genome organisation of globin genes was investigated by analysis of total cellular DNA by SOUTHERN-blotting (JEFFREYS & FLAVELL 1977) or by structural analysis of cloned chromosomal DNA-fragments.

The chromosomal alpha and beta globin gene of Balb/c mice have been cloned in a lambda vector (LEDER et al. 1978, KONKEL et al. 1978, NISHIOKA & LEDER 1979). The adult Balb/c mice coordinately expresses two beta globin genes encoding the

two polypeptides beta d major and beta d minor in a ratio of 4 to one (CURTIS personal communication). The two genes are located on two distinct EcoRI fragments and are embedded in non homologous segments of DNA. The two genes are closely linked and presumably evolved from a common ancestral gene 50 million years ago (KONKEL et al. 1979). They differ in 9 out of 146 amino acids and cross hybridize efficiently. A single type of alpha gene was reported and completely sequenced by NISHIOKA et al. 1979. Alpha and beta genes are located on different chromosomes and are thought to originate from a common ancestral gene by duplication 450 million years ago. Two intron sequences at similar relative positions were reported in all three genes. A recombinant plasmid containing mouse fetal globin sequences was described by FANTONI et al. 1979.

A restriction map for the rabbit beta globin gene deduced directly from rabbit chromosomal DNA was presented by JEFFREYS & FLAVELL 1977. The gene was cloned by Van den BERG et al. 1978 and LACY et al. 1979 and completely sequenced by Van OUYEN et al. 1979 and HARDISON et al. 1979. Based on hybridization properties one single beta globin gene is predominantly detected in rabbit chromosomal DNA. LACY et al. 1979 reported a set of cloned overlapping fragments containing a cluster of 4 closely linked beta-like globin genes in rabbit. Two of them showed hybridization to a human gamma-globin probe. The rabbit beta globin gene cluster resembles the situation found in man, except that the human delta gene does not share homologies with the corresponding rabbit gene.

A restriction map of the beta globin gene cluster of human chromosomal DNA was presented by BERNARDS et al. 1979. A DNA fragment containing the human beta globin gene cluster was cloned and characterised by LAWN et al. 1978. The four closely linked genes are located in the order 5' to 3': G-gamma/A-gamma/delta/beta. The human alpha globin genes are located on a

different chromosome but are duplicated and lie close together as reported by ORKIN 1978. Human fetal globin genes were characterised by RAMIREZ et al. 1979. Molecular cloning of human epsilon genes was reported by PROUDFOOT & BARALLE 1979.

A chromosomal chicken beta globin gene was partially characterised by DODGSON et al. 1979. He found the beta globin gene closely linked to a beta like embryonic gene.

All globin genes reported so far have the same general structure. The coding sequence is arranged in three non contiguous blocks interrupted by two intron sequences, a smaller one of 110 to 130 base pairs close to the 5' end and a larger one of 150 to 800 base pairs in the middle of the gene. The middle part of the large intron is the least conserved part of the closely related globin sequences (Van Ooyen et al. 1979, KONKEL et al. 1979, NISHIOKA & LEDER 1979).

Some aspects of gene regulation have been reported in the globin gene system. During the development of the fetus a switch in human globin genes was reported (WEATHERALL et al. 1979). In very young embryos up to three month old, the epsilon genes are actively expressed. Later in fetal life and until birth, the A-gamma and G-gamma globin genes are activated. Within 6 month after birth, the expression of A-gamma/G-gamma genes is switched off and the beta and delta globin genes are expressed. Beta and delta globin chains are present in a ratio of 40 to one (WOOD et al. 1978). The current view is, that two switches in beta like globin gene expression take place during human development (epsilon-gamma-beta), while only one is observed in rabbit and mouse.

A number of globin anemias and thalassemias have been investigated on the chromosomal DNA level. A disease known as Hb-LEPORE or delta/beta-thalassemia was investigated on the DNA level by MEARS et al. 1978 and FLAVELL et al. 1978. A deletion of about 8000 base pairs joined the 5' part of the delta gene to the 3' part of the beta gene. In this case an

abnormal hemoglobin is formed by fusion of the delta and beta genes. Such gene fusions are thought to arise from unequal crossing over during meiosis.

OLD et al. 1978 studied cases of beta-zero-thalassemias by hybridization of total poly-A RNA to normal cDNA. In some cases he was not able to detect beta globin mRNA, but in other cases low levels of beta globin mRNA were found, which failed to direct the synthesis of beta globin in a cell-free protein synthesis system.

By comparing globin sequences of various species and studying abnormal globin phenotypes, information can be obtained about the functional significance of certain DNA sequences. In addition the mechanisms of evolution, selection and mutation fixation can be studied by direct effects on the DNA.

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MATERIALS:

Acrylamide and N'N-methylene-bisacrylamide were from Serva
Agarose, Type II medium EEO (SIGMA)

Dimethylsulfate (Fluka puriss.)

Ficoll (Pharmacia)

Formamide (Fluka)

Hydrazin (Eastman Kodak)

Millipore filters of 0.45 μ pore size were from Millipore Co.

N,N,N',N' tetramethylene-diamide (TEMED) from Eastman Kodak

Phenol (Siegfried) was freshly distilled and stored at -20°C .

Polyvinylpyrrolidone (SIGMA)

Pyperidine(Fisher Scientific Co.)

^{125}I odine carrier free was from Eidg. Institut für Reactor-
forschung Würenlingen.

^{32}P -ATP and ^{32}P -dCTP 0.5 mCi/nMol were obtained from NEN

^{32}P -gamma ATP 7 mCi/nMol was a gift of C. WEISSMANN

Preparations of rabbit globin mRNA were gifts of T. STAEHELIN
and of mouse mRNA by R. WILLIAMSON.

ENZYMES:

KpnI restriction endonuclease was a gift of R. FLAVELL

EcoRI

W. BOLL

AluI and HindII

S. CLARKSON

BspI

A. KISH

BamHI

W. MUELLER

BglII and HhaI were purified according to BICKLE et al. 1977

Enzymes of sufficient quality for DNA sequencing were ob-
tained from Biolabs.

Reverse transcriptase was a gift of P. CURTIS purified
according to BALTIMORE & SMOLER 1972.

Lambda exonuclease and terminal deoxynucleotidyl transferase
were gifts of W. BOLL.

Nuclease S₁ 60 000 U/mg, purified according to WIEGAND et al. 1975

RNase A (Worthington) was dissolved in 1 x TNE, 5 mg/ml and heated for 10 minutes at 70°C.

Pronase (Calbiochem) was dissolved 20 mg/ml in 10 mM Tris pH 7.5, 5 mM EDTA and predigested for 2 hours at 37°C.

E. coli DNA polymerase I (Biolabs) 8000 U/mg

T₄ polynucleotide kinase (Biolabs) 16000 U/mg

Calf intestine alkaline phosphatase (Boehringer) 400 U/mg

SOLUTIONS AND BUFFERS:

1 x SSC 0.15M NaCl/ 0.015M sodium citrate pH 7.0

1 x TNE 0.1M NaCl/ 50mM Tris.HCl pH 7.5/ 5mM EDTA

S₁-mix 30mM sodium acetate pH 4.5/ 1mM ZnSO₄/ 0.2M NaCl

Table 1: INCUBATION BUFFERS FOR RESTRICTION ENZYMES

Buffer	Tris.HCl	MgCl ₂	DTT	NaCl	Enzymes
Y-0	10mM pH 7.5	6 mM	1 mM	-	AvaI,II/BglI
Y-10	"	"	"	10 mM	CauI,II/HaeII/HpaI,II MboII/BclI/BglIII/TaqY PvuII/RspI
Y-50	"	"	"	50 mM	AluI/BspI/EcoRII/HinII,III
Y-100	"	"	"	100 mM	BamHI/EcoRI/PstI/SalI
Y-150	"	"	"	150 mM	HaeIII/HhaI/MboI/PvuI XhoI/XbaI
Z-10	10mM pH 8.5	"	"	10 mM	SmaI

ELECTROPHORESIS BUFFERS:

Tris-acetate pH 7.8: 50mM Tris(Base)/ 20mM sodium acetate/
2mM EDTA

Loening E pH 7.8: 36mM Tris(Base)/ 30mM NaH₂PO₄/ 1mM EDTA

Tris-borate pH 8.3: 50mM Tris(Base)/ 50mM boric acid/ 2mM EDTA

Tris-borate pH 8.3: 90mM Tris(Base)/ 90mM boric acid/ 2mM EDTA

Staining solution for gels: 0.5 µg/ml ethidium bromide in

1 x electrophoresis buffer.

Loading dye: 20% Ficoll/ 100mM EDTA/ 0.01% bromophenol blue.

GEL ELECTROPHORESIS:

Agarose gels were run as described by Mc DONNEL et al. 1977. Agarose was dissolved in 1 x electrophoresis buffer, autoclaved or simply boiled, solidified in small horizontal trays and electrophoresed at 10 Volts/cm.

Table 2: Agarose gels and buffer systems:

Length of DNA fragment	% agarose	buffer system
50 000 - 2 000 bp	0.5%	Loening E or Tris-acetate pH 7.8
30 000 - 500 bp	1%	" "
5 000 - 200 bp	2%	" "
1 500 - 100 bp	2%	Tris-borate 90 mM, containing 0.5 µg/ml ethidium bromide

An example of a large scale preparative agarose gel is described in the results section part A.

Acrylamide gels were prepared as described by LOENING 1969 and PEACOCK & DINGMAN 1978.

5% acrylamide gels were prepared as follows: 5g acrylamide and 1.6 g N'N-methylene-bisacrylamide were dissolved in 100 ml 1 x electrophoresis buffer and degassed. 50 µl TEMED and 1 ml 10% ammonium persulfate were added and poured between vertical glass plates 30cmx20cmx2mm.

10% gels contained 10 g acrylamide and 3.2 g N'N-methylene-bisacrylamide.

Table 3: Acrylamide gels and buffer systems:

Length of DNA fragment	% acrylamide	buffersystem
3000 - 100 bp	5%	Loening E (samples must be free of salts and proteins)
1500 - 50 bp	5%	Tris-borate 90 mM
500 - 10 bp	10%	Tris-borate 90 mM

DNA sequencing gels:

Stock solutions of the composition as listed in table 4 were prepared in 1 liter of the indicated 1 x electrophoresis buffer.

Table 4: Stock solution for sequencing gels

% acrylamide	bis-acryl	urea	buffer
20% 193 g/l	6.6 g/l	420 g/l	50 mM Tris-borate
12% 120 g/l	6.5 g/l	420 g/l	"
5% 48 g/l	2.0 g/l	420 g/l	"

120 ml of stock solution was degassed, mixed with the amounts of TEMED and 10% ammonium persulfate indicated in Table 5 and poured between 40cmx30cmx1mm vertical glass plates. 4mmx1mm slotformers were inserted between the plates.

Table 5: Amounts of catalysts per 120 ml sequencing gel

% acrylamide	TEMED	10% ammonium persulfate
20%	20 μ l	0.7 ml
12%	25 μ l	0.8 ml
5%	50 μ l	1.0 ml

The solution polymerized slowly at room temperature; the gels stored overnight before use. They were prerun for 5 to 10 hours at 700 Volts and the sample wells were rinsed with buffer to remove urea before applying the samples. 12% gels with runs for 2, 7, 14, and 22 hours allowed the determination of the sequence of 150 or even 200 nucleotides.

EXTRACTION OF DNA FRAGMENTS FROM GELS:

DNA fragments were eluted from agarose gels by passing the gel through a syringe into 10 - 20 volumes of extraction buffer containing 150 mM NaCl, 10 mM EDTA, 10 mM Tris pH 7.5, shaking the suspension at room temperature overnight and passing through a 3D-glass filter by vacuum. The DNA was concentrated by passing it through a small DE-52 column, which was washed with 2 volumes of extraction buffer. DNA was eluted with 1.5 M NaCl, 2 mM EDTA, 10 mM Tris pH 7.5. The eluate was extracted with phenol/chloroform and three times with ether, and the DNA precipitated with ethanol.

The same procedure was followed for the extraction of DNA-

fragments from acrylamide gels except that the gel suspension was centrifuged for 10 minutes at 10 000 rpm and the supernatant collected. Phenol/chloroform extraction was omitted.

TRANSFER OF DNA FRAGMENTS FROM AGAROSE GELS TO MILLIPORE FILTERS:

The method was described by SOUTHERN 1975 and JEFFREYS & FLAVELL 1977. The agarose gel was placed for 10 minutes in a solution 0.1 M HCl, 15 minutes in 0.5 M NaOH and 15 minutes in 2.5 M NaCl/0.5 M Tris pH 7.5. Two sheets of wet 3MM-paper were placed on a glass plate, dipping on two sides into a buffer reservoir containing 20 x SSC. The arrangement was covered with a saran foil and a window of the size of the gel cut into the foil. The gel was placed on the window with a Millipore filter wetted in 2 x SSC on top. Two sheets of wet 3MM paper and several sheets of dry filterpaper were added and changed from time to time. A weight of 0.5 kg was placed on top of the whole arrangement. The transfer usually took 24 hours. The Millipore filter was removed, washed for 15 minutes in 2 x SSC and dried in a vacuum oven for 2 hours at 80°C. Hybridization was done as described for colony hybridization.

IODINATION OF mRNA:

The method was described by COMMERFORD 1971 and OROSZ & WETMUR 1974. The reaction contained 0.5 - 2 µg of mRNA in a total volume of 10 µl, 0.25 M ammonium acetate pH 5, 1 mM tellurium chloride and 1 µCi carrier free ¹²⁵Iodine. Incubation was for 20 minutes at 60°C in a ventilated hood. The reaction was adjusted to 0.25 M Tris pH 8.5, 25 mM sodium sulfite and incubated for 20 minutes at 60°C. Material excluded from Sephadex G-100 was ethanol precipitated and purified on a 5 - 20% neutral sucrose gradient.

Long RNA (larger than 200 - 300 bases) leads to high background in filter hybridizations and was fragmented as follows: The RNA was dissolved in 50 mM sodium bicarbonate, incubated for 30 minutes at 50°C, ethanol precipitated and resuspended in

0.1 x TNE. Specific activities of $10^7 - 10^8$ cpm per μg of RNA were obtained.

DNA LABELING BY NICK TRANSLATION:

The method was described by RIGBY et al. 1977. The reaction contained 50 mM Tris-HCl pH 8.0, 5 mM MgCl_2 , 2.5 mM DTT, 50 $\mu\text{g/ml}$ BSA, 1 mM unlabeled dGTP and dTTP, 0.2 - 2 μg of DNA template, 3U of E. coli DNA polymerase I (containing traces of DNase) and 50 μCi of ^{32}P -dATP and ^{32}P -dCTP in a total volume of 20 μl . Incubation was for 60 to 90 minutes at 15°C . The reaction was stopped by phenol/chloroform extraction in 1 x TNE. Material excluded from Sephadex G-100 was ethanol precipitated and re-suspended in H_2O . Specific activities of $2 - 10 \times 10^6$ cpm per μg of DNA were obtained.

5'-END LABELING OF NUCLEIC ACIDS BY T_4 -POLYNUCLEOTIDE KINASE:

The method was essentially described by MANIATIS et al. 1976 and MAXAM & GILBERT 1977. The DNA was cleaved with a restriction endonuclease, extracted with phenol/chloroform and ether, and passed over a small column of Chelex in 50 mM Tris-HCl pH 7.5. 10 - 20 μg of DNA in a total volume of 300 to 400 μl were incubated at 65°C with 0.4U of bacterial alkaline phosphatase (Worthington) for 30 - 60 minutes. The reaction mixture was adjusted to 1 x TNE, extracted with phenol/chloroform and three times with ether. The DNA was concentrated on a small DE-52 column and ethanol precipitated.

The phosphatase-treated DNA was resuspended in 25 μl kinase buffer containing 50 mM Tris-HCl pH 9.5, 2 mM DTT, 1 mM spermidine, 10 mM MgCl_2 , 2.5 U of polynucleotide kinase and 20 pmoles (50 μCi) of gamma ^{32}P -ATP. The reaction was incubated for 20 minutes at 37°C and stopped by the addition of 50 μl of 4 M ammonium acetate, 25 mM EDTA. Material excluded from Sephadex G-50 was ethanol precipitated and resuspended in 0.1 x TNE or H_2O .

ENZYMATIC SYNTHESIS OF cDNAs:

The principle of the procedure was described by ROUGEON et al. 1975 and EFSTRATIADIS et al. 1976.

The assay contained in a total volume of 20 μ l: 40 mM Tris-HCl pH 8.0, 25 mM NaCl, 5 mM MgCl₂, 0.5 mM DTT, 0.2 μ g of oligo dT-primer, 1 mM unlabeled deoxy-triphosphates and 10 U of reverse transcriptase. The mixture was incubated for 90 minutes at 37°C. The reaction was stopped by phenol/chloroform extraction in 1 x TNE. Material excluded from Sephadex G-100 was collected and ethanol precipitated. The cDNA was resuspended in 0.5 M KOH and incubated for 40 minutes at room temperature and finally purified on a neutral sucrose gradient 5 - 20 %.

The second strand was synthesized following the same assay conditions. After ethanol precipitation, the DNA was dissolved in 25 μ l of 1 x S₁-mix (see buffers) and incubated for 30 minutes at 37°C with 5 U of S₁ nuclease. The reaction was stopped by phenol/chloroform extraction in 1 x TNE. Material excluded from Sephadex G-100 was purified on a 5 - 20 % neutral sucrose gradient.

LAMBDA EXONUCLEASE TREATMENT:

5' overhanging ends of DNA fragments are very poor substrates for terminal transferase. After mild lambda exonuclease treatment a dramatic improvement of elongation was observed (RADDING et al. 1971). The reaction mixture contained: 100 mM sodium glycinate adjusted to pH 9.5 with NaOH, 20 mM MgCl₂, 50 μ g/ml of BSA, 50 to 100 μ g/ml DNA and 0.5 U lambda exonuclease per μ g of DNA. The mixture was incubated for 60 minutes at 0°C. The reaction was stopped by phenol/chloroform extraction in 1 x TNE and the solution three times extracted with ether.

ELONGATION BY TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE:

The method was essentially as described by JACKSON et al. 1972, LOBBAN & KAISER 1973 and MANIATIS et al. 1976 and in addition by ROYCHOUDHURY et al. 1976.

The reaction mixture contained for elongation with dA residues: 1 mM dATP, 100 mM sodium cacodylate adjusted to pH 7.2 with NaOH, 10 mM NaH_2PO_4 , 5 mM MgCl_2 , 50 $\mu\text{g/ml}$ of BSA, 50 to 100 $\mu\text{g/ml}$ of DNA and 3 U enzyme per μg of DNA. Incubation was for 45 - 60 minutes at 37°C .

The assay for elongation with dT residues contained: 1 mM dTTP, 100 mM sodium cacodylate adjusted to pH 7.2 with NaOH, 2.5 mM CoCl_2 , 50 $\mu\text{g/ml}$ of BSA and 0.6 U enzyme per μg of DNA. Incubation was for 45 minutes at 37°C . The reaction was stopped by phenol/chloroform extraction in 1 x TNE, the mixture three times ether extracted and dialysed against 1 x TNE for two days. On average 200 - 300 dA respectively dT residues were added.

TRANSFECTION:

The method (Van den BERG et al. 1978) is a modification of the procedure described by CURTISS III et al. 1977.

E. coli X1776 was grown at 37°C in 1% tryptone, 0.1% glucose, 0.1% yeast extract, 0.8% NaCl, 0.03% CaCl_2 and 100 μg per ml of diaminopimelic acid. At an optical density of $A_{650\text{nm}} = 0.7$ the cells were chilled, centrifuged, washed successively with 10 mM NaCl, 100 mM CaCl_2 and resuspended in 1/25 of the original culture volume of 100 mM CaCl_2 . 0.1 ml CaCl_2 -treated cells was added to 0.1 ml of annealed DNA (2 $\mu\text{g/ml}$) (elongated DNA fragments were annealed in 1 x TNE at 50 - 100 $\mu\text{g/ml}$ at 60°C for 2 hours, at 40°C for 2 hours and at room temperature for one hour) in 100 mM NaCl, 100 mM Tris-HCl pH 7.5, 40 mM CaCl_2 and 40 mM MgCl_2 , and incubated for 20 minutes on ice and for 10 minutes at 20°C . 0.2 ml of tryptone broth was added and the bacteria were plated on agar containing 25 $\mu\text{g/ml}$ kanamycin sulfate, 10 $\mu\text{g/ml}$ nalidixic acid and 100 $\mu\text{g/ml}$ diaminopimelic acid. About 10 000 colonies per μg of elongated rabbit DNA were found.

MILLIPORE REPLICA TECHNIQUE AND COLONY HYBRIDIZATION:

This technique was developed by van Ooyen and was described by van den Berg et al. 1978. Dry prewashed Millipore filters were placed on top of the colonies, carefully removed and incubated on fresh agar plates (colony-bearing side up, as drawn in Scheme 3 results part A: cloning procedure). The membranes were placed on 3MM paper, soaked with 0.1 M HCl for 10 minutes, on 0.5 M NaOH for 10 minutes, on 1 M Tris-HCl pH 7.5 for 2 minutes and on 2.5 M NaCl/0.5 M Tris-HCl pH 7.5 for 10 minutes (as described by Grunstein & Hogness 1975). The filters were air dried and incubated in a solution containing 1 mg/ml proteinase K in 2 x SSC for 15 minutes at room temperature. Filters were washed in ethanol and chloroform and dried for 2 hours at 80°C in a vacuum oven. For hybridization with ³²P-DNA the filters were preincubated for 4 to 6 hours at 37°C in a solution containing 50% formamide, 0.65 M NaCl, 100 µg/ml denatured salmon sperm carrier DNA, 0.02% polyvinyl pyrrolidone, 0.02% Ficoll, 0.02% BSA (Denhardt 1966). The filters were individually hybridized for 15 hours at 37°C under paraffin oil in about 1 ml of the same solution containing 1 - 3x10⁵ cpm of ³²P-labeled denatured DNA. The filters were successively washed in 50% formamide/5xSSC for 15 minutes and in 0.2xSSC at 37°C for 30 minutes. The dry filters were autoradiographed for several days at -70°C with an Ilford Fast Tungstate intensifying screen (Swanstrom & Shank 1978).

For hybridization with RNA no prehybridization was necessary. 50 - 100 µg/ml yeast RNA carrier were included in the hybridization mixture. The background was efficiently removed by treatment with pancreatic RNase 50 µg/ml in 2 x SSC for 20 minutes at room temperature.

ISOLATION OF PLASMID DNA:

A modification of the method described by Currier & Nester 1976 was used. A clone was picked from an agar plate and incubated overnight in 50 ml tryptone medium. 10 ml of this culture was

used to inoculate one liter tryptone medium containing the supplements and the desired antibiotics. The culture was grown by shaking in a 2 liter Erlenmeyer flask at 37°C until an apparent $A_{650} = 0.9$ was reached. The cells were harvested at 4000 rpm for 15 minutes, resuspended in fresh medium containing in addition 170 µg/ml chloramphenicol and shaken at 37°C overnight. The cells were killed in the morning by addition of 10 ml of chloroform and shaken vigorously for 10 minutes. The cells were centrifuged at 4000 rpm for 15 minutes at 4°C, washed in 20 mM Tris-HCl pH 7.5/1 mM EDTA, centrifuged again and resuspended in 30 ml of 50 mM Tris-HCl pH 7.5/25% sucrose at 0°C. 30 mg of lysozyme were added and mixed. 5 minutes later the solution was adjusted to 100 mM EDTA and kept at 0°C for 5 minutes. The solution was brought to 2% SDS, mixed and immediately adjusted to 1 M NaCl. The solution was mixed and kept at 0°C for 24 hours. The precipitate was pelleted in a SW 27 rotor at 20 000 rpm for 30 minutes at 4°C, and the supernatant was collected and extracted with one volume phenol/chloroform and once with one volume of chloroform. The phases were separated by centrifugation at 5000 rpm for 5 minutes. The solution was treated with 20 µg/ml pancreatic RNase for 60 minutes at 37°C. 0.2 volumes of 5 M NaCl and 0.3 volumes of 30% polyethylene glycol 6000 were added, mixed and kept at 4°C overnight. The precipitate was collected by centrifugation at 10 000 rpm for 15 minutes at 0°C and dissolved in 15 ml of 50 mM Tris-HCl pH 8.0/5mM EDTA. 5 M NaOH was added to pH 12.4, while stirring with a magnetic stirrer; the glass electrode was calibrated at pH 11 before use. The pH was kept at 12.4 for 10 minutes at room temperature. 1 M Tris-HCl pH 7.5 was added to a pH value of 8.5 and the solution kept for 3 minutes at room temperature. 1/10 volume of 5 M NaCl was added and vigorously mixed with 1 volume of phenol for 5 minutes. The phases were separated by centrifugation for 10 minutes at 10 000 rpm at 4°C. The aqueous phase was adjusted to 0.3 M sodium acetate and precipitated

with 2.5 volumes of ethanol at -20°C overnight. The plasmid DNA was collected by centrifugation at 10 000 rpm for 10 minutes and resuspended in 0.2 - 1 ml of 0.1 x TNE. The last step of purification was a neutral sucrose gradient 5 - 20%.

RESTRICTION MAPPING:

DNA was restricted, 5'-end labeled and cleaved asymmetrically by a second restriction endonuclease. Fragments labeled at one end were isolated from agarose or acrylamide gels and subjected to partial cleavage with various restriction enzymes as described by SMITH & BIRNSTIEL 1976. In this way the distance of 3 to 5 restriction sites from the labeled end could be mapped. Similarly prepared 5'-end labeled fragments were the substrates for the DNA-sequencing reactions. Partial restriction cleavages were obtained as follows: 20 μl reactions with the appropriate buffer containing 2000 - 5000 cpm of 5'- ^{32}P -labeled fragment and 1 μg of calf thymus carrier DNA were run in parallel with 1, 0.3 and 0.1 units of enzyme for one hour at 37°C . The reactions were stopped by the addition of loading dye and directly applied to the gel.

DNA SEQUENCING SOLUTIONS:

Methylation buffer (DMS buffer):

50 mM sodium cacodylate adjusted to pH 8 with NaOH, 10 mM MgCl_2 , 0.1 mM EDTA.

DMS stop solution:

1 M Tris-acetate pH 7.5, 1.5 M sodium acetate pH 7.5,

1 M beta-mercapto-ethanol, 50mM magnesium acetate, 1 mM EDTA
0.5 mg per ml yeast carrier RNA.

Hydrazin stop solution:

0.3M sodium acetate pH 6.0, 10 mM EDTA, 10 mM magnesium acetate,
25 μg per ml yeast carrier RNA.

Yeast carrier RNA:

Yeast RNA was dissolved at 10 mg per ml in 1 x TNE, extracted three times with phenol/chloroform and once with chloroform

and three times with ether. The RNA was ethanol precipitated and resuspended in 10 mM Tris-HCl pH 7.5 at 10 mg per ml.

Sonicated calf thymus carrier DNA:

The DNA (SIGMA) was dissolved at 2 mg/ml in 10 mM Tris-HCl pH 7.5, sonicated with a Branson sonifier, microtip, level 6 for 10 x 30 seconds in 30 second intervals, filtered through Millipore 0.45 μ m pore size, adjusted to 1 x TNE, extracted with phenol/chloroform, once with chloroform and three times with ether. The DNA was ethanol precipitated and dissolved in 10 mM Tris-HCl pH 7.5 at 4 - 5 mg/ml.

Pyridin-formiate 1 M pH 2.0:

To 4 ml of conc. formic acid 70 ml of H₂O were added and the pH adjusted to 2.0 by dropwise addition of pyridin. The final volume was adjusted to 100 ml with H₂O.

Siliconized Eppendorf tubes:

The tubes were filled with a solution of 5% dimethyldichlor-silan in carbontetrachloride and kept at room temperature for one hour. The solution was removed by suction, the tubes rinsed three times with H₂O and dried in vacuum.

DNA SEQUENCING, SPECIFIC DEGRADATIONS (MAXAM & GILBERT 1977):

G-reaction:

The DNAs were processed in siliconized Eppendorf tubes containing 2.5 μ l of ³²P-DNA, 1 μ l sonicated calf thymus carrier DNA (4 mg/ml), 200 μ l DMS buffer and 1 μ l dimethyl sulfate. After 3 - 4 minutes incubation at 30°C the reaction was stopped by the addition of 50 μ l of DMS stop solution. 750 μ l of cold ethanol were added and precipitated in an ethanol dry ice bath for 10 minutes. The DNA was pelleted in a small Eppendorf centrifuge for 5 minutes. The supernatant was carefully removed by a drawn-out pasteur pipette and the pellet resuspended in 250 μ l of 0.3 M sodium acetate buffer (pH 6.0) and reprecipitated by the addition of 750 μ l of cold ethanol. The pellet was washed once with ethanol and dried in vacuum.

G plus A reaction:

The reaction contained 3.5 μ l of 32 P-DNA, 1 μ l carrier DNA (4 mg/ml), 16.5 μ l of H₂O and 2 μ l of 1 M pyridin-formiate buffer (pH 2.0). After 60 - 70 minutes of incubation at 30°C the reaction mixture was frozen and lyophylized in high vacuum, 25 μ l of H₂O were added and the sample was lyophylized once more.

C plus T reaction:

The assay contained 5 μ l of 32 P-DNA, 1 μ l carrier DNA (4 mg/ml), 20 μ l of H₂O and 30 μ l of hydrazine, and was incubated for 10 minutes at 20°C. 200 μ l of hydrazine stop solution were added and the DNA was precipitated with 750 μ l ethanol as described for the G-reaction. The pellet was resuspended in 250 μ l of 0.3 M sodium acetate buffer (pH 6.0) and precipitated by the addition of 750 μ l of ethanol. This cycle was repeated once more. Finally the DNA was washed in cold ethanol and dried in vacuum.

C-reaction:

The assay contained 5 μ l of 32 P-DNA, 1 μ l carrier DNA (4 mg/ml), 20 μ l of 5 M NaCl and 30 μ l of hydrazine and was incubated for 12 minutes at 20°C. The mixture was further processed exactly as described for the C plus T reaction.

All four reaction mixtures were treated with piperidine as follows: The dried DNAs were dissolved each in 25 μ l of 1 M piperidine, individually transferred into glass capillaries and carefully sealed. The reactions were heated for 30 minutes at 90°C, cooled down and transferred into siliconized Eppendorf tubes. The samples were frozen and lyophylized. 25 μ l of H₂O were added and the samples again lyophylized. This cycle was repeated once more. The dried DNAs were finally resuspended carefully in 10 - 20 μ l of formamide dye mixture (90% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol blue, 1 mM EDTA), heated for 60 seconds at 90°C, chilled on ice for 60 seconds and loaded immediately onto a prerun sequencing gel as described

under gel electrophoresis. The gels were usually transferred onto Alumina plates covered with Alufoil and sandwiched together with an X-ray film and an Ilford Fast Tungstate intensifying screen between the Alu-plates, pressed together by metal clamps and exposed at -70°C in a light tight box.

S₁-MAPPING OF DNA FRAGMENTS:

Closely related DNAs can be characterised by heteroduplex formation and after successive treatment with S₁-nuclease the size of the products can be analysed by gel electrophoresis, if one of the components was specifically labeled. The assay is a modification of the method described by BERK & SHARP 1977 and was applied to the characterisation of mouse 15s beta-globin cDNA plasmids by heteroduplex formation with Bam-cleaved, 5'-³²P-labeled, cloned chromosomal DNA. (See Scheme 4). The assay was carried out as follows: unlabeled HhaI-cleaved cDNA plasmids and ³²P-labeled chromosomal DNAs were filtered over a small column of Chelex. 25 µl of hybridization mixture in a siliconized Eppendorf tube contained 50% formamide, 5 x SSC, 0.1% SDS, 250 µg/ml denatured salmon sperm carrier DNA, 0.5 - 1 µg unlabeled and 1 - 10 ng (2000 - 5000 cpm) of labeled DNA. One drop of paraffin oil was added, the mixture was heated for 2 minutes at 90°C and incubated overnight at 37°C . The paraffin oil was removed by three ether extractions and to each tube were added 200 µl of S₁-mix (see solutions and buffers) and 5 µl of S₁ nuclease (150 units), and the tubes were vigorously vortexed. The assay mixture was incubated for 60 minutes at 30°C , once more ether extracted and ethanol precipitated. The pellet was resuspended in 10 µl of formamide dye, heated for 60 seconds at 90°C , chilled for 60 seconds on ice and loaded immediately on a 5% acrylamide gel containing 7 M urea as described under gel electrophoresis.

BACTERIAL STRAINS AND MEDIA:

E. coli K 12 strain HB 101 was originally obtained from H. BOYER (BOYER et al. 1969) and used for transfection with well characterised components and for plasmid DNA production. The strain has the following genetic characteristics:

$r_B^-/m_B^-/leu^-/gal^-/pro^-/lac^-/str^R/recA^-$
contains the region hapB-thi of E. coli B/r

The strain was grown on tryptone-medium containing:

1% Bacto-tryptone, 0.1% glucose, 0.1% yeast extract, 0.8% NaCl and 0.03% $CaCl_2$.

E. coli K 12 strain X1776, in conjunction with the plasmid pCRI represents a certified EK2 cloning system (CURTISS III et al. 1977) and was used for the cloning of eukaryotic genes. The strain is deficient in cell wall synthesis and can not survive in natural environment, extreme sensitivity to detergents was reported.

Genotype: $F^-/tonA53/dapD8/minA1/supE42/oms-2/thyA57/metC65/$
 $oms-1/minB2/rfb-2/nalA25/cycB2/cycA1/hsdR2/$
 $lambda^-/del.40(gal-uvrB)/del.29(bioH-asd)$

The strain is resistant against nalidixic acid 50 - 100 $\mu g/ml$ cycloserin 15 $\mu g/ml$ and trimethoprim.

It was usually grown on tryptone-medium supplemented by 100 $\mu g/ml$ of diaminopimelic acid, 50 $\mu g/ml$ thymidin and 10 $\mu g/ml$ nalidixic acid.

SAFETY PROCEDURES:

All operations required for the cloning of eukaryotic genes were carried out in a laminar flow biological safety cabinet located within a negative air pressure containment facility. All material was stored for a limited time, disposable labware was used and destroyed routinely by autoclaving. The possible biohazard was further reduced by cloning a partially purified fraction of eukaryotic DNA.

PLASMIDS:

pCRI:

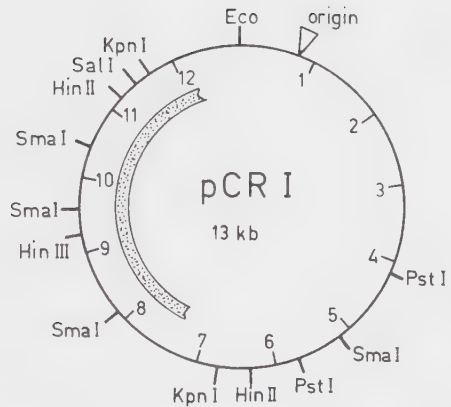
Certified NIH
cloning vector

Reference:

BOLIVAR et al. 1977

WICKENS et al. 1979

 kanamycin
resistance gene



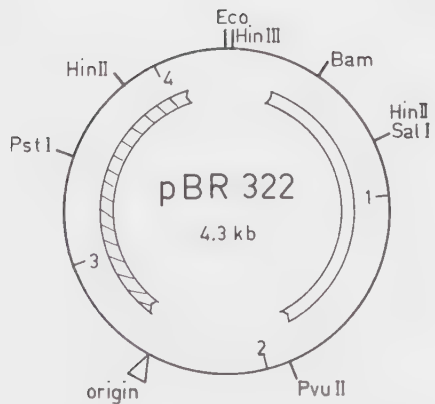
pBR 322:

Certified NIH
cloning vector

Reference:

SUTCLIFFE 1978

 ampicillin
resistance gene
 tetracyclin
resistance gene



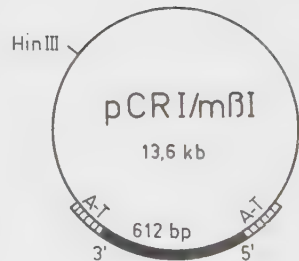
p β G:

Recombinant plasmid consisting of pMB9 and a cDNA copy of rabbit beta-globin mRNA, constructed by MANIATIS et al. 1976 (resistant to 50 μ g/ml tetracyclin)



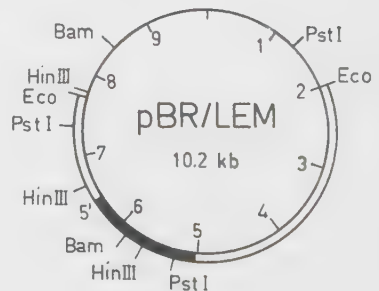
pCRI/m β I:

Recombinant plasmid containing a cDNA copy of mouse beta-globin mRNA, characterised by MANTEI 1979 (personal communication) (resistant to 50 μ g/ml kanamycin)



pBR/LEM:

Recombinant plasmid containing a chromosomal 6 kb EcoRI fragment of Balb/c mouse beta major globin, cloned in λ gtWES λ B by LEDER et al. 1977, and transferred into the EcoRI-site of pBR322 by W. BOLL. (resistant to 50 μ g/ml ampicillin)



ABBREVIATIONS:

AluI, AvaII	}	Abbreviations for restriction endonucleases see Reference: ROBERTS R., Gene <u>4</u> , 183 (1978)
BamHI, BglII, BspI		
EcoRI, HhaI, HinII		
HinIII, Hinf, HphII		
KpnI, MboII, PstI		
SalI, TaqI		
BSA		bovine serum albumin
cpm		counts per minute
cDNA		complementary DNA
DNA		deoxy-ribonucleic acid
dA, dT		deoxy-adenosin, deoxy-thymidine
DTT		dithiothreitol
EDTA		ethylene diamine tetraacetate
pCR _I , pBR322, p 8 G	}	Abbreviations for plasmid DNAs see under plasmids in the Materials and Methods section
pCR _I ^m 8 1, pBR/LEM		
bp		base pair
kb		kilo bases
RNA		ribonucleic acid
SDS		sodium-dodecyl-sulfate
TEMED		N,N,N',N',-tetramethylene diamide
Tris		tris-(hydroxymethyl)-aminomethane
ul		microliter 10 ⁻⁶ liter
ug		microgram 10 ⁻⁶ gram
ng		nanogram 10 ⁻⁹ gram
pg		picogram 10 ⁻¹² gram
pMol		pico Mol
mM		milli molar
125 _I -mRNA		radio-Iodine labeled messenger RNA
32 _P		radioactive phosphor isotope
10s, 15s		sedimentation index obtained by gradient centrifugation

RESULTS PART A: CONSTRUCTION OF A BACTERIAL PLASMID CONTAINING THE RABBIT BETA GLOBIN GENE

Partial purification of rabbit beta globin DNA and construction of hybrid plasmids

In collaboration with J. van den BERG and A. van OUYEN a cloning system was set up to isolate the rabbit beta globin gene. We decided to cleave the rabbit DNA with the restriction endonuclease KpnI, which generates a 5.1 kb fragment containing beta globin sequences (JEFFREYS & FLAVELL 1976) and to purify the DNA by gel electrophoresis. A fraction enriched about 50-fold in beta globin sequences was used for cloning.

Hybrid plasmids were obtained by annealing of dT-elongated pCRI plasmid DNA to dA-elongated rabbit DNA and transfected into E. coli X1776. The single steps of this approach are summarized in Scheme 2.

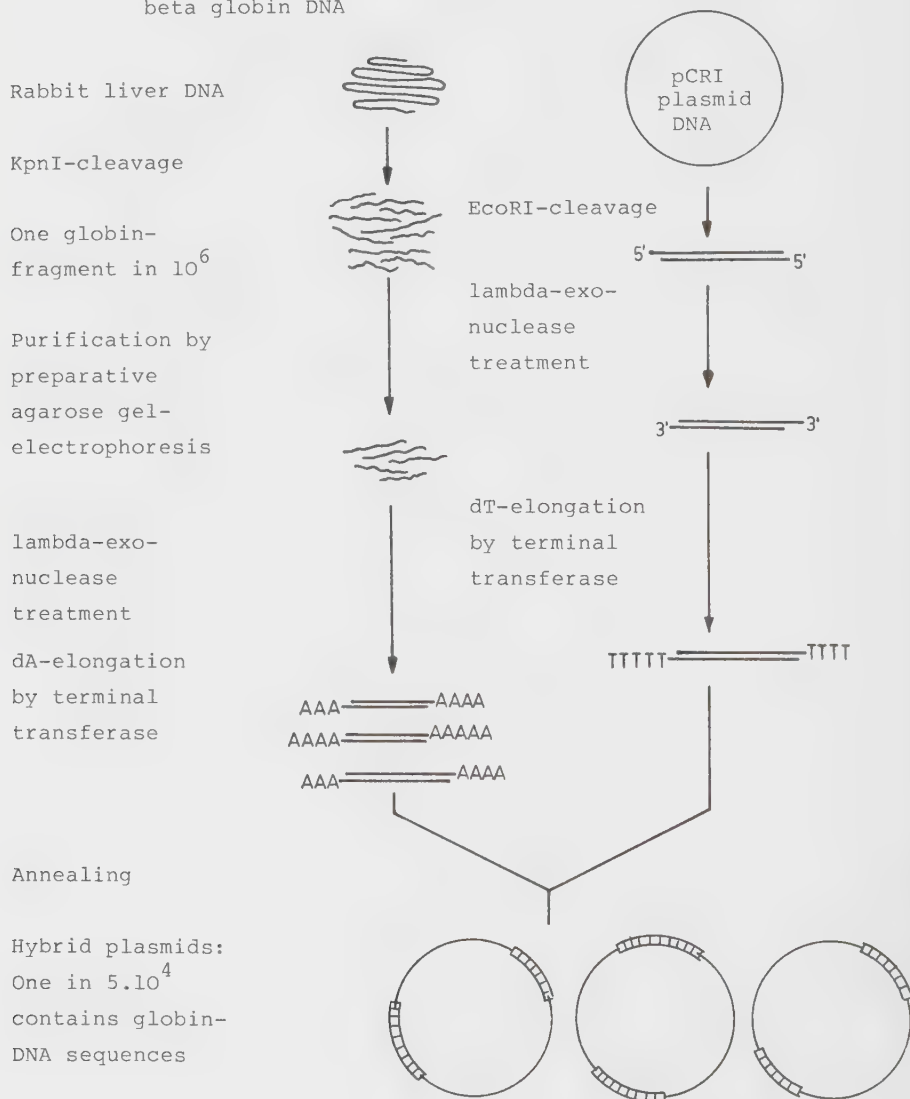
300 to 500 single colonies were grown per plate on N-agar containing kanamycin and transferred by a replica plating technique to a Millipore filter. The replica filters were placed on new agar plates and incubated overnight. The colonies were lysed on the filters as described by GRUNSTEIN & HOGNESS 1975 and hybridized to a radioactively labeled globin specific probe. X-ray films were exposed to the Millipore filters and positive colonies were identified on the original master plates as shown in Scheme 3.

All the single steps are described in detail in the following chapters and in the Materials and Methods section.

Partial purification of KpnI-cleaved rabbit DNA by preparative agarose gel electrophoresis

Chromosomal DNA was extracted from livers of inbred Vienna white and Alaska rabbits according to BLIN & STAFFORD 1976. About 50 mg of high molecular weight DNA were extracted from 30 g of fresh livers.

SCHEME 2: Construction of hybrid plasmids containing
a fraction of partially purified rabbit
beta globin DNA



SCHEME 3: Analysis of clones

Hybrid plasmids are trans-
fected into *E. coli* X1776

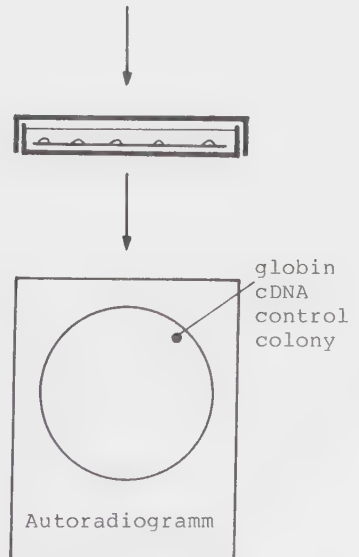
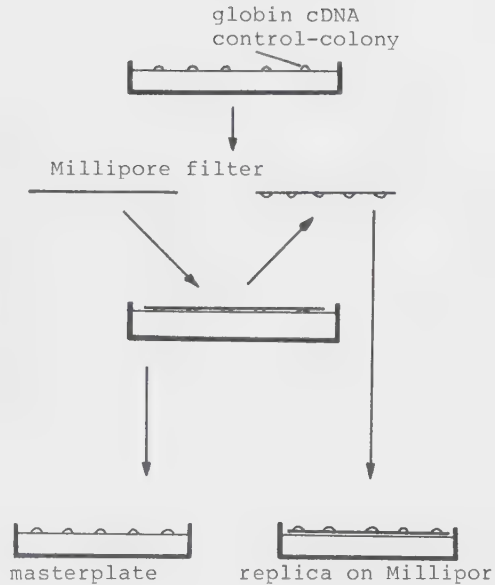
Transfectants are plated
on about 500 masterplates
containing 300-500 colonies
each

Replicas on Millipore
filters are prepared
and incubated on fresh
agarplates

Millipore filters are
hybridized to a ^{32}P -labeled
restriction fragment of
beta globin DNA

X-ray film is exposed to
Millipore filters

Positive colonies are
identified on the original
masterplates



The DNA was cleaved with the restriction endonuclease KpnI. Cleavage was checked for completeness by adding pCRI plasmid DNA which contains KpnI sites to an aliquot of the incubation mixture and monitoring the cleavage on a 1% agarose gel.

The cleaved rabbit DNA was fractionated on a preparative agarose gel with the dimensions 30 x 20 x 1.6 cm essentially as described under Materials and Methods. Exposure of the KpnI-cleaved DNA to ethidium bromide and UV light was avoided. Marker fragments were run on the same gel alongside the preparative slots, cut away after the run and stained separately. The region in the preparative gel corresponding to about 3 to 6 kb, where the beta globin KpnI fragment was known to run (JEFFREYS & FLAVELL 1977) was sliced into 1 cm strips and the DNA was eluted as described under Materials and Methods. Aliquots of the eluted DNAs were assayed as described in the next chapter.

Assay of globin DNA-containing fractions,

Estimation of the purification

The results of a typical assay are summarized in Figure 1. 4 μ g aliquots of the partially purified DNA fractions were run on analytical agarose gels (Figure 1A). The DNA was denatured in the gel, transferred to a Millipore filter according to SOUTHERN 1975 and hybridized to 32 P-labeled nick translated p β G plasmid DNA according to JEFFREYS & FLAVELL 1977, as shown in Figure 1B. The fraction in lane 2, but none of the others, showed a radioactive band at about 5.1 kb. Labeled plasmid DNA without globin insert did not give rise to a band in a control hybridization (results not shown). In the preparative experiment 3.5 mg of DNA were cleaved and fractionated as described above. About 20 μ g were recovered in the globin DNA-containing fraction. Assuming a DNA recovery of 60 - 70 % from the gel, we estimate a purification of 50 to 100 fold.

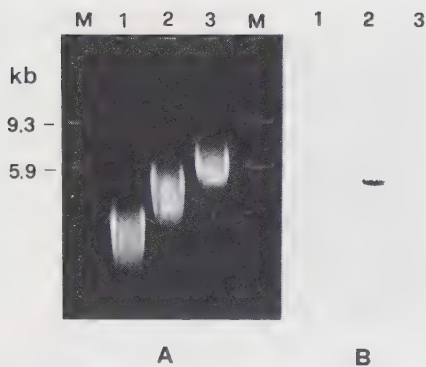


Figure 1:

Analysis of partially purified beta-globin DNA

- A) 0.8% agarose gel, stained in ethidium bromide and photographed under short wave UV-light
Lanes 1,2 and 3 represent 4 μ g aliquots of DNA fractions purified on a preparative agarose gel as described under Materials and Methods
M=size markers: ϕ 29 DNA digested with EcoRI.
- B) The DNA in gel A) was transferred to a Millipore filter (SOUTHERN 1975) and hybridized to 32 P-labeled p β G plasmid DNA (specific activity 1 - $4 \cdot 10^7$ cpm/ μ g). Fraction 2 contains a globin specific band.

In the starting material we expected one globin fragment in $5 \cdot 10^5$ to 10^6 (HOFSTETTER thesis 1976) and after purification, one in 10^4 to $5 \cdot 10^4$. In the actual cloning experiment the larger number was closer to reality.

This assay is only semi-quantitative and quite slow, but has the advantage that the result is clearly visualized by a band in the expected position. Cloned cDNAs have proven to work as highly specific probes to detect single genes (JEFFREYS & FLAVELL 1976, BREATHNACH et al. 1978, LOMEDICO et al. 1979). Nick translated labeled probes with a specific activity of 10^7 cpm/ μ g DNA are able to detect between 5 and 10 pg of specific sequences. This amount is present in about 10 μ g of total DNA (JEFFREYS & FLAVELL 1977).

Preparation of hybrid DNA clones; use of 125 I-mRNA for colony hybridization leading to identification of ribosomal DNA clones

The partially purified KpnI-cleaved beta-globin-containing DNA was elongated with dA residues using deoxynucleotidyl transferase as described by CLARKE & CARBON 1975. In a preliminary experiment, incubations for different time periods, yielding different length of dA-tails, were dialyzed extensively against 1 x TNE at 4°C and hybridized at a one to one molar ratio to dT-elongated pCRI plasmid DNA. Hybrid plasmids were transfected into E. coli X1776. The conditions yielding the highest rate of transformants, about 10 000 per μ g of hybrid-plasmid DNA, were established by J. van den BERG and are described in the Methods section. About 10 000 clones were picked onto 40 plates and hybridized to (alpha plus beta) globin mRNA labeled with 125 Iodine, as described in the Methods section. The idea to use labeled globin mRNA was favoured initially because the globin cDNA plasmid contained homologous vector sequences to the plasmid used for cloning. The approach to purify a globin specific restriction fragment was considered to be more troublesome, quantitatively limiting and possibly not of sufficient specificity. With the improvement of purifi-

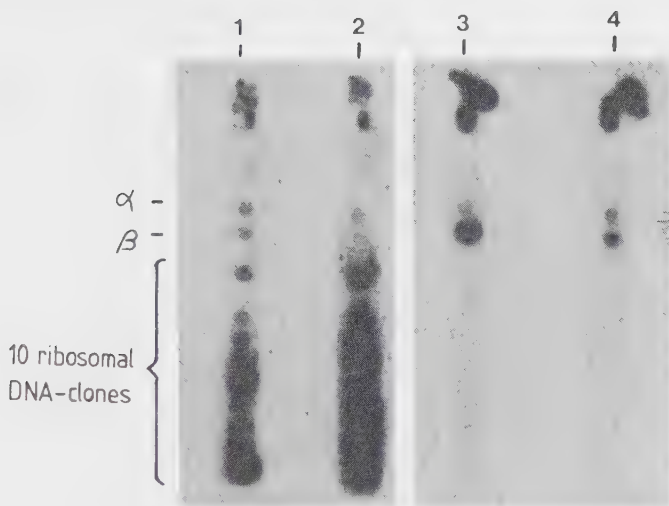


Figure 2:

Colony hybridization of ribosomal DNA-clones

1-4 Colony filters were prepared according to GRUNSTEIN & HOGNESS 1975, containing one alpha and one beta control colony and 10 test colonies each and were hybridized separately to the indicated probes as described in the Materials and Methods section.

1 Hybridized to 18s ^{32}P -labeled ribosomal RNA

2 Hybridized to 28s ^{32}P -labeled ribosomal RNA

3 Hybridized to alpha globin ^{125}I -labeled mRNA

4 Hybridized to beta globin ^{125}I -labeled mRNA

(specific activities: $5 \cdot 10^6 - 10^7$ cpm/ μg)

The test colonies hybridized exclusively to 18s and 28s ribosomal RNA and not to the globin specific probes.

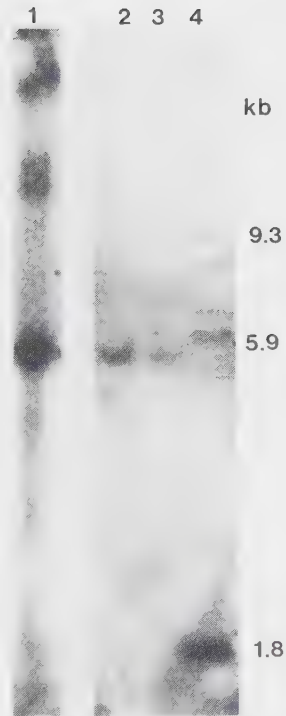
cation methods for restriction fragments and labeling procedures and with the failure to detect a globin specific gene using globin ^{125}I -mRNA (this chapter), this second approach became the method of choice (next chapter).

The Millipore filters were processed as described by GRUNSTEIN & HOGNESS 1975. Positive colonies were detected with a frequency of about one in 800. 10 colonies were picked from the original masterplates and further analysed. The unexpectedly high yield of positive colonies suggested that the hybridization probe may have been contaminated by ribosomal RNA, leading to the identification of ribosomal DNA clones. 10 clones were therefore analysed using ribosomal RNA probes and repurified alpha and beta globin mRNA probes (Figure 2). Different intensities of hybridization to 18s and 28s ^{32}P -labeled ribosomal RNA and no hybridization to purified alpha and beta ^{125}I -labeled globin mRNA was observed. The specific alpha and beta probes were prepared by hybridization and elution of mRNA from filters containing alpha or beta globin plasmid DNA, as described by CURTIS et al. 1978. When these probes were tested on alpha and beta DNA-filters, they usually hybridized with about 80% specificity. In the experiment of Figure 2 the hybridization appears rather unspecific, possibly due to different reaction conditions which led to a high background. The faint hybridization of ^{32}P -labeled 18s and 28s ribosomal RNA to alpha and beta globin plasmid containing colonies may also reflect an unspecific background. Additional analysis in Figure 3: total KpnI-cleaved rabbit DNA and an aliquot of the partially purified fraction were run on a 1% agarose gel, transferred to a Millipore filter and hybridized to ^{32}P -labeled ribosomal RNA or ^{125}I -labeled globin mRNA. A band at about 5500 base pairs was visible in all DNA-probes, demonstrating the presence of ribosomal DNA in the partially purified DNA fraction as well as the presence of ^{125}I -ribosomal RNA in the ^{125}I -mRNA preparation.

Figure 3:

Detection of ribosomal DNA in KpnI-cleaved total rabbit DNA and in a DNA fraction enriched for beta-globin DNA.

10 μ g of KpnI-cleaved rabbit DNA and 4 μ g of the partially purified globin DNA (see Figure 1, lane 2) were run on a 1% agarose gel, transferred to a Millipore filter and individually hybridized to the indicated probes, as described in the Materials and Methods section.



Lane 1: KpnI-cleaved rabbit DNA, hybridized to 32 P-labeled 18s and 28s ribosomal RNA

Lane 2: KpnI-cleaved rabbit DNA, hybridized to 125 I-labeled globin mRNA

Lane 3: partially purified rabbit DNA (Figure 1, lane 2), hybridized to 125 I-globin mRNA

Lane 4: Size markers: 32 P-labeled ϕ 29 phage DNA digested with EcoRI restriction endonuclease.

The beta-globin band migrating at about 5100 base pairs was not visible in this blotting due to the low specific activity of the ^{125}I -mRNA.

The conclusions of this experiment can be summarized as follows: all steps of the procedure are working in principle, as demonstrated by the isolation of chromosomal ribosomal DNA-clones. However, for the screening of beta-globin clones a highly specific probe for hybridization is needed. In a first attempt to improve the specificity of the hybridization ^{125}I -mRNA was purified over oligo-dT cellulose. Moreover, a 500-fold excess (50-100 $\mu\text{g/ml}$) of cold competitor ribosomal RNA was added to the hybridization mixture. However, the hybridization to rDNA-containing clones could not be competed out completely and we finally considered another possibility.

Isolation and identification of a rabbit beta-globin gene,
Comparison with the restriction map of rabbit chromosomal DNA

A new cloning experiment was started, using a recently described probe which hybridized to rabbit beta globin DNA specifically. Plasmid pBG (MANIATIS et al. 1976) was cleaved by HaeIII-endonuclease and the 333 base pair fragment containing beta-globin coding sequences only, was purified on a preparative 5% polyacrylamide gel, as described under Materials and Methods, and ^{32}P -labeled by nick translation as described by RIGBY et al. 1976. More than 50 000 single colonies were screened by hybridization to the new probe (Van den BERG et al. 1978) as described in the preceding chapter. Positive clones were identified on X-ray film, picked from the original master plate and rehybridized. One rabbit chromosomal beta-globin DNA-containing clone was detected and analysed by restriction mapping. The map of rabbit chromosomal beta-globin DNA and its flanking regions as deduced by JEFFREYS & FLAVELL 1977 by single gene hybridization and SOUTHERN-blotting using the restriction endonucleases EcoRI, BamHI, BglII and PstI was

confirmed by the restriction map of the cloned chromosomal DNA fragment as shown in Figure 4. An intron of about 650 base pairs is present between the BamHI and EcoRI sites. Moreover a smaller intron of about 125 base pairs was localised closer to the 5' end by partial restriction mapping (SMITH & BIRNSTIEL 1976) from the ^{32}P -labeled Bam-site using the enzyme MboII. This intron sequence was too small to be picked up by the method of JEFFREYS & FLAVELL. A similar small intron at a comparable position was also localised in the chromosomal mouse beta-globin gene (KONKEL et al. 1978). The presence of intron sequences is not peculiar to the globin gene system. On the contrary, introns have been demonstrated in a number of chromosomal genes in higher eukaryotes: ovalbumin (BREATHNACH et al. 1978, CATTERALL et al. 1978), immunoglobulin genes (TONEGAWA et al. 1978), ovomucoid genes (DUGAICZYK et al. 1978), lysozyme gene (SIPPEL et al. 1978), alpha globin (LEDER et al. 1978), human beta and delta globins (MEARS et al. 1978, FLAVELL et al. 1978) and silkfibroin (TSUJIMOTO et al. 1978). No introns were found in a tRNA_{met} gene of *Xenopus laevis* (CLARKSON et al. 1978) and in a cytochrome C gene of yeast (MONTGOMERY et al. 1978). The physiological meaning of intron sequences in higher eukaryotes can not be explained based on the data collected so far.

A 15s beta-globin precursor RNA to 10s mature mRNA was demonstrated in mouse Friend cells (CURTIS et al. 1978) and in mouse embryos (KINNIBURGH et al. 1978). The question was asked whether extra sequences at the 5' or 3' end would be present. By R-loop mapping (TILGHMAN et al. 1979) the presence of the large intron could be demonstrated. Part B of this work shows that both the large and the small introns are present in the 15s transcript.

A partial DNA sequence analysis of the regions close to the borders of the large intron of the rabbit beta-globin gene is shown in Figure 5. The complete DNA sequence including stretches of the flanking sequences was determined by Van Ooyen et al. 1979 and is shown in Figure 6.

Figure 4:

Restriction maps of cloned rabbit beta-globin chromosomal DNA, genomic DNA and cDNA.

The restriction sites for the enzymes BamHI, EcoRI, BglII and PstI in the cloned DNA were mapped as described by Van den BERG et al. 1978 and corresponded well to the map of genomic DNA as determined by JEFFREYS & FLAVELL 1977, The same large intron of about 650 base pairs was found between the BamHI and the EcoRI sites. An additional small intron of about 125 base pairs was localized close to the 5' end (Van den BERG et al. 1978). The map of the cDNA was determined by MANIATIS et al. 1976.

The designation Z-pCRI/Rchr β G-1 means:

Z: Zürich, R: rabbit, chr: chromosomal

β : beta, G: globin

-  coding sequences
-  flanking regions and plasmid DNA
-  intervening sequences (introns)

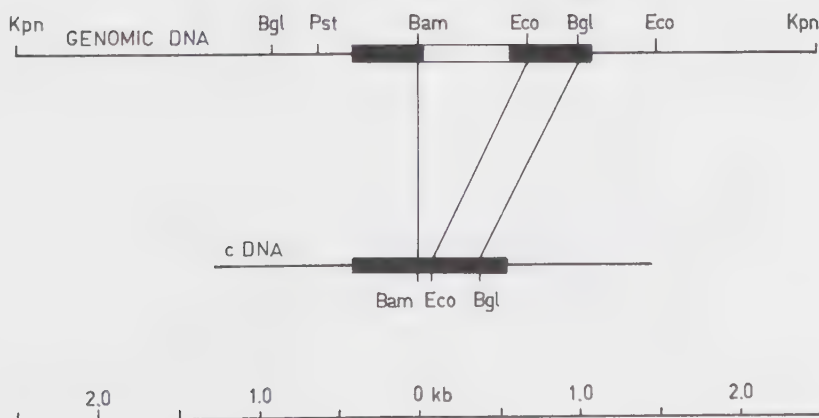
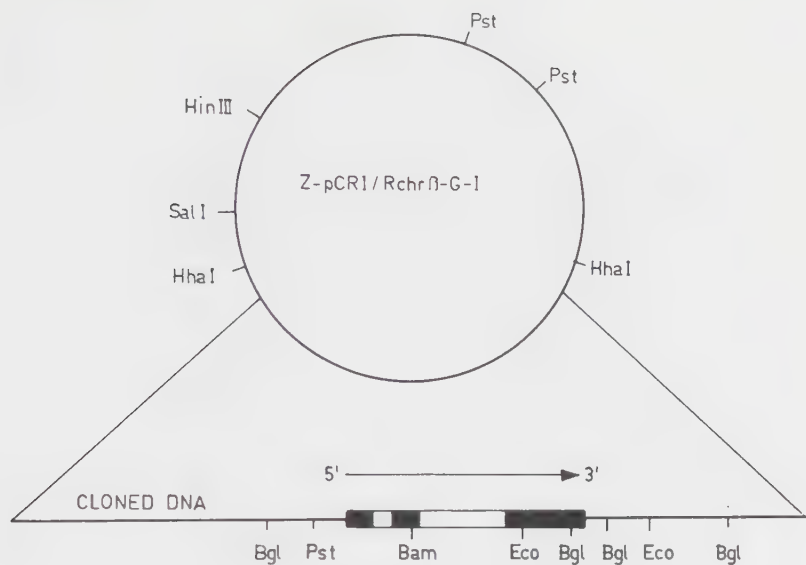
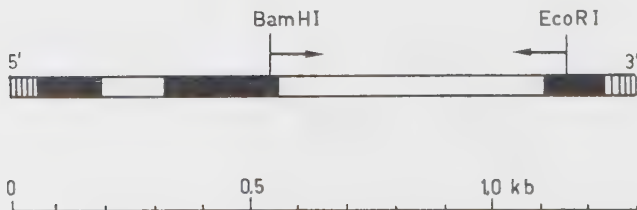
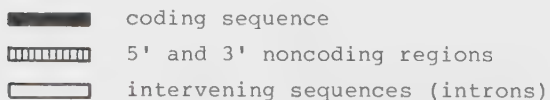


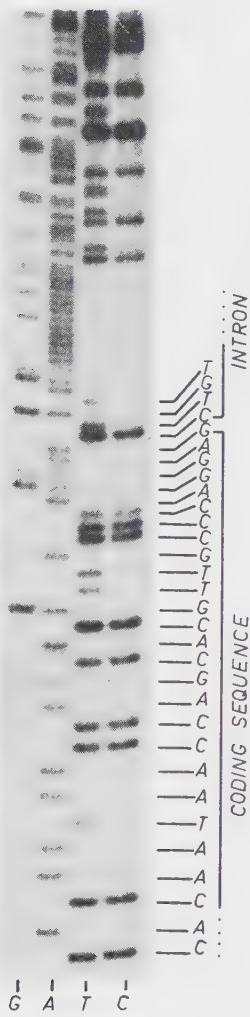
Figure 5:

Nucleotide sequence at the borders of the large intron towards the coding sequences of the cloned rabbit beta-globin gene

The indicated fragment labeled at the BamHI or at the EcoRI site respectively was subjected to the base specific cleavage reactions described by MAXAM & GILBERT 1977 and run on a denaturing 12% polyacrylamide gel as described in the Materials and Methods section and by Van den BERG et al. 1978. The positions of the sequenced parts in the cloned gene are marked in the following small map by arrows.



EcoRI



BamHI

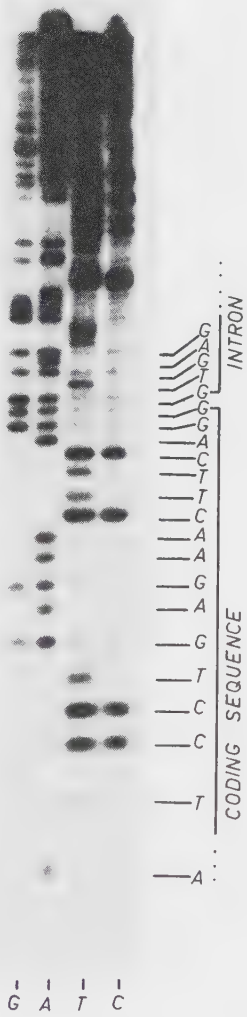


Figure 6:

The complete nucleotide sequence of a cloned chromosomal rabbit beta-globin gene and its flanking sequences

Position one corresponds to the capped nucleotide of the mRNA. Cap and pA designate the positions of the cap and poly A tail respectively.

Nucleotides marked by a dot have been deduced from the recognition sequence of a restriction site and the amino acid sequence in that region.

Five nucleotides marked by an asterisk have not been reliably determined.

All the details were described by Van Ooyen et al. 1979.

Figure 6:

The complete nucleotide sequence of a rabbit beta-globin gene (Van Ooyen et al. 1979)

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TAGCAATTAGTACTGCTGGTATGGTCTGGGAGATACATAGAGGAGGCTGAGTCTGCAGACTCTTAAGCATTGCCATAAGTCCCAA
-220 -210 -200 -190 -180 -170 -160 -150 -140

GGACAGGGGTGCTGTCTACACCCAGACCTCACCTCGAGAGCCACACCTGGTGTGGCCAACTACACACGGGTAGGGATTACATAGT
-130 -120 -110 -100 -90 -80 -70 -60 -50

TCAGGAATTGGGCATAAAGGCGAGCAGGGGAGCTGCTGCTTACACTTGGCTTTGACACAACCTGTGTTCATTGCAATCCCCAAAACA
-40 -30 -20 -10 0 10 20 30 40

GACAGAAATGGTCACTGTCCAGTGAGGAGAAGTCTGCGGTCACTGCCCTGTGGGGCAAGGTGAATGTGAAGAAGTTGGTGGTGAAGCC
50 60 70 80 90 100 110 120 130
Met Leu His Leu Ser Ser Glu Glu Lys Ser Ala Val Thr Ala Leu Tyr Gly Lys Val Asn Val Glu Glu Val Gly Glu Ala

CTGGGCAAGTTGGTATCCTTTTACAGCACAACCTTAATGAGCAGATAGAACTGGTCTGTGAGAAACAGAGTAGTGCCTGCTTTCTG
140 150 160 170 180 190 200 210 220
Leu Gly Ar

CCAGGTGCTGACTTCTCCCCGGGCTGTTTTCATTTTCTAGGGTCTGGTGTGCTACCCATGGACCAAGGTCTCTCAGTCCCTTT
230 240 250 260 270 280 290 300 310
GLeu Leu Val Val Tyr Pro Tyr Thr Gln Arg Phe Phe Glu Ser Phe

GGGACCTGCTCTGCAAAATGCTGTATGAACAATCCTAAGGTGAAGGCTCATGGCAAGAAGTGTGCTGCTTCAGTGAAGGCTG
320 330 340 350 360 370 380 390 400
Gly Asp Leu Ser Ser Ala Asn Ala Val Met Asn Asn Pro Lys Val Lys Ala His Gly Lys Lys Leu Ala Ala Phe Ser Glu Gly Leu

AGTCACCTGGACAACCTCAAAGGCACCTTTGCTAAGCTGAGTGAAGTGCACCTGTGACAAGCTGCACGTGGATCTCGAAGCTTCAGGGTG
410 420 430 440 450 460 470 480 490
Ser His Leu Asp Asn Leu Lys Gly Thr Phe Ala Lys Leu Ser Glu Leu His Cys Asp Lys Leu His Val Asp Pro Glu Asn Phe Arg

AGTTGGGACCTTGATTGTTCTTTCTTTTGGCTATTGTAATTCATGTTATATGAGGGGGCAAGTTTCAGGGGTGGTGTAGA
500 510 520 530 540 550 560 570 580

ATGGGAGATGTCCTTGTATCACCATGGACCTCATGATAATTTGTTCTTTCACCTTCTACTGTTGACCAACCATGTCTCTCTT
590 600 610 620 630 640 650 660 670

ATTTCTTTTCATTTCTGTAACCTTTTCGTAACTTAGCTTGCAATTTGTAACGAATTTTAAATTCACCTTTGTTATTGTCAGAT
680 690 700 710 720 730 740 750 760

TGTAACTACTTCTTAATCACTTTTTTCAAGGCAATCAGGGTATATATTGTTACTTCAGCACAGTTTAGAGAACAACTGTTATA
770 780 790 800 810 820 830 840 850

ATTAATGATAAGGTAGAATAATTCTGCATATAAATCTGGCTGGGTGAAATATTCTTATGAGAAACAACTACATCCCTGGTCATC
860 870 880 890 900 910 920 930 940

ATCTGCTTTCTCTTATGGTACAATGATATACACTGTTGAGATGAGGATAAAACTCTGAGTCAAAACGGGCCCTCTGCTAAC
950 960 970 980 990 1000 1010 1020 1030

CATGTTCTGCTCTCTCTTTTCTACAGCTCCTGGGCAACGTGCTGGTATTGTGTGCTCATCATTTTGGCAAAAGATTCACTCCT
1040 1050 1060 1070 1080 1090 1100 1110 1120
Leu Leu Gly Asn Val Leu Val Ile Val Leu Ser His His Phe Gly Lys Glu Phe Thr Pro

CAGGTGCAAGCTGCTATCAGAAGTGGTGGCTGGTGGTGGCAATGCCCTGGCTCACAAATACACCTGAGATCTTTTCCCTTGCCAAA
1130 1140 1150 1160 1170 1180 1190 1200 1210
Gln Val Gln Ala Ala Tyr Gln Lys Val Val Ala Gly Val Ala Asn Ala Leu Ala His Lys Tyr His

AATTATGGGACATCATGAAGCCCTTGAGCATCTGACTCTGGCTAATAAGGAAATTTATTTTCATTGCTATAGTGTGTGAAATTT
1220 1230 1240 1250 1260 1270 1280 1290 1300

TTGTGCTCTCACGGAAGGACATATGGGAGGCAAAATCATTTAAACATCAGAAATGATTTGTTTAAAGTTTGGCAACATATGCC
1310 1320 1330 1340 1350 1360 1370 1380 1390

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RESULTS PART B: CHARACTERISATION OF HYBRID PLASMIDS CONTAINING COMPLEMENTARY DNA COPIES OF THE MOUSE 15S BETA GLOBIN PRECURSOR RNA

Construction of mouse 15s-beta-globin cDNA plasmids

The 10s beta-globin mRNA is synthesized via a 15s precursor RNA. CURTIS et al. 1977 isolated the 15s beta-globin precursor RNA from erythroleukemic Friend cells and showed that the 5' end was capped and the 3' end contained a 150 nucleotide poly(A) tract. When this RNA was first discovered there were speculations about the existence of extra sequences at the 5' and 3' ends. When it was later established that the beta globin genes contained a small and a large intron, the question arose as to whether the extra sequences of the 15s precursor were internal. TILGHMAN et al. 1978 showed by an R-looping experiment between 15s precursor RNA and chromosomal DNA that the two structures lined up completely when inspected under the electron microscope. KINNIBURG et al. 1978 and SMITH & LINGREL 1978 analysed the fragments resulting from RNase cleavage of heteroduplex molecules between 15s precursor and 10s mRNA and concluded that both the small and the large introns are transcribed into the precursor RNA. Definitive evidence for a structure containing both introns is presented in the following chapters by the construction and analysis of cDNA plasmids from 15s precursor RNA. Most of the starting material was provided by P. CURTIS, who initiated these experiments.

15s precursor RNA was isolated from erythroleukemic Friend cells as described in detail by CURTIS et al. 1977 and TILGHMAN et al. 1978 and converted into double-stranded complementary DNAs using reverse transcriptase. The cDNA was elongated with dA residues using deoxynucleotidyl terminal transferase and hybridized to EcoRI-cleaved dT-elongated pCRI plasmid DNA as described in detail by MANIATIS et al. 1976 and in the Materials and Methods section. The hybrid plasmids were introduced into E. coli X1776, and kanamycin resistant clones

where isolated and tested by colony hybridization (GRUNSTEIN & HOGNESS 1975) to ^{125}I -labeled mouse 10s globin mRNA. 200 positive clones were streaked out on new filters and hybridized to a ^{32}P -labeled restriction fragment comprising essentially only the large intron of cloned chromosomal beta major globin. The probe was prepared by cleaving the beta globin chromosomal hybrid plasmid pBR/LEM with BamHI and PstI, purifying the 600 base pair fragment on an agarose gel, and labeling it by nick translation, as described in the Materials and Methods section. 12 clones which hybridized to the large intron probe were picked, plasmid DNA was isolated and recloned in E. coli HB101. The colonies were rechecked in duplicate by colony hybridization to the probe described above. Figure 7 shows an autoradiogram of this experiment, which includes a positive control (E. coli HB101 containing the chromosomal mouse beta globin plasmid pBR/LEM) and negative controls (host strain HB101, the same strain containing the vector plasmid pCRI and a colony containing a mouse 10s beta globin cDNA plasmid pCRI/m β 1). The clones were designated Z-pCRI(EcoRI)/Mc-15s- β G-1 through -12, or M15s- β G-1 etc. for short. 11 clones showed hybridization to the specific fragment indicating homologies to the large intron sequence of the beta globin chromosomal gene. Clone M15s- β G-7 did not hybridize and the plasmid insert represented a small fragment of 10s mRNA as discovered later by restriction mapping and a partial sequence analysis (results not shown).

Characterization of hybrid plasmids

Plasmid DNA of each of the 12 clones was prepared, cleaved with the restriction endonuclease HhaI and run on a 1.5% agarose gel as described in the Materials and Methods section. In each case, the insert is contained in the largest HhaI fragment, flanked by the dAT tails and a 725 and 625 base pair fragment of pCRI on either side (Figure 8). Clone M15s- β G-1 contained an almost full length copy of 15s beta major globin

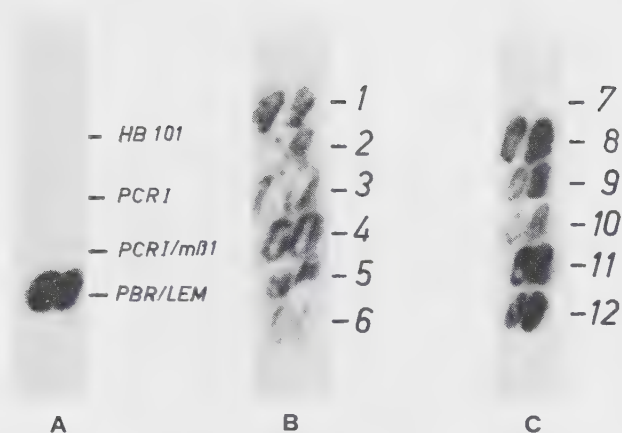


Figure 7: Colony hybridization of M15s- β G clones

Filters of duplicate colonies were prepared according to GRUNSTEIN & HOGNESS 1975 and hybridized to the 600 bp BamHI-PstI fragment containing the large intron of the mouse beta major globin gene. The probe was 32 P-labeled by nick translation (spec. activity $5 \cdot 10^6$ cpm/ μ g) as described in the Materials and Methods section.

A controls: from top to bottom:

- host strain HB101
- strain HB101 containing the vector plasmid pCRI
- strain HB101 containing a cDNA plasmid representing mouse 10s beta globin mRNA, pCRI/m β 1
- the positive control was the cloned chromosomal beta major globin gene, pBR/LEM

B and **C**: the M15s- β G clones numbered from 1 to 12, all of which gave a positive response with exception of number 7.

as shown by the restriction mapping and nucleotide sequence determination described below. Clones M15s- β G-5,6 and 9 were identified as beta minor globin species by restriction mapping and partial sequence determination. The second large HhaI fragment which shows up in clone M15s- β G-6 must be considered as an artefact; since the data deduced by restriction mapping and sequence determination around the BamHI site, as described below, were in agreement with the beta minor type described by KONKEL et al. 1979. The possibility that a mixture of two different plasmids was isolated or that a mutation had occurred at an HhaI site can not be excluded. Four clones were of special interest, because the cDNA insert contained the large but not the small intron. Two of these clones, number 3 and number 6 were characterised in more detail; they are thought to represent splicing intermediates of a beta major and a beta minor species respectively. A summary of the structures based on the data collected from all 12 clones is presented in Figure 18.

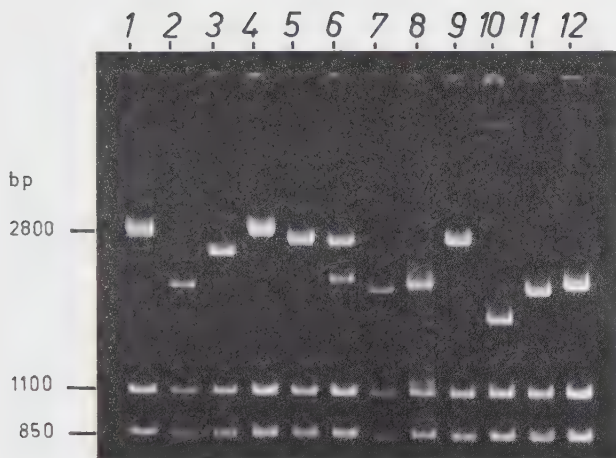


Figure 8: HhaI-cleavage of M15s- β G plasmid DNAs

2 μ g of plasmid DNA of each of the M15s- β G clones (numbered from 1 to 12) were cleaved with the restriction endonuclease HhaI and electrophoresed on a 1.5% agarose gel, stained in ethidium bromide and photographed under a short wave UV lamp as described in the Materials and Methods section.

The largest fragment in each digestion gives an estimate of the size of the inserted DNA including dAT linkers.

Restriction map and nucleotide sequence analysis of clone M15s- β G-1, comparison with the cloned chromosomal mouse beta major globin gene.

Plasmid DNA of the clone M15s- β G-1 was cleaved at the unique BamHI site, ³²P-labeled at the 5' termini and digested with HhaI. Two labeled fragments of 1350 and 1400 bp respectively, were isolated by agarose gel electrophoresis and subjected to partial restriction mapping (SMITH & BIRNSTIEL 1976). In addition, M15s- β G-1 was digested with HhaI and the largest fragment was isolated, 5'-terminally labeled and cleaved with HinIII. The two resulting fragments of 1200 and 1550 bp were mapped in the same way. Altogether 25 restriction sites were mapped in the 15s part (Figure 9); they had the same relative distances as the corresponding beta major gene cloned by KONKEL et al. 1979. The restriction map of the non-sequenced plasmid segments is not completely certain. The nucleotide sequence of the insert of clone M15s- β G-1 was determined according to the sequencing strategy outlined in Figure 10, following the protocol of MAXAM & GILBERT 1977. All the labeled fragments are listed in Table 6. The complete sequence is presented in Figure 11. As compared to the sequenced beta major gene (KONKEL et al. 1979, Van OUYEN et al. 1979), 52 nucleotides were missing from the region corresponding to the 5' terminal end of the mRNA and 233 nucleotides were missing at the 3' end. Figure 11 shows the presumed complete structure of 15s-beta major cDNA; the missing segments (underlined) are taken from the sequence of the beta major gene (KONKEL et al. 1979). It should be noted that the revised sequence reported by KONKEL et al. 1979 for the chromosomal beta globin DNA differs in some regards from the 15s cDNA sequence determined in this work. Some of these discrepancies are believed to be due to errors in the sequence of KONKEL et al. 1979, others may be due to polymorphism. In total 1106 nucleotides of the 15s cDNA were compared with corresponding chromosomal sequences: (Figures 11 and 12).

- (1) Residue 308 (coding sequence) is G in the chromosomal sequence and A in the 15s cDNA. This is an authentic difference (Figure 12, A and B) and results in a change of an amino acid residue (Gly in the chromosomal DNA, Arg in the 15s cDNA).
- (2) As compared to the 15s cDNA sequence, the chromosomal sequence has an additional T between residues 765 and 766 (in the large intron). Figure 12, C and D shows that this too is an authentic difference between the two sequences.
- (3) The region between residues 836 and 850 has 4 T's in the cDNA which were not reported for the chromosomal DNA. Since the T's are poorly resolved in this part of the sequence gel (Figure 12, E), it is likely that the chromosomal sequence was misread by KONKEL et al. 1979. The chromosomal sequence within this region was not rechecked by us.
- (4) As compared to the 15s-cDNA sequence (Figure 12, F), the chromosomal sequence (KONKEL et al. 1979) has an additional G residue between position 1084 and 1085, following the PstI site and is not in agreement with the chromosomal sequence determined by N. MANTEI (Van OUYEN et al. 1979). There is no discrepancy between the 15s cDNA and the chromosomal sequence determined by N. MANTEI at this position.

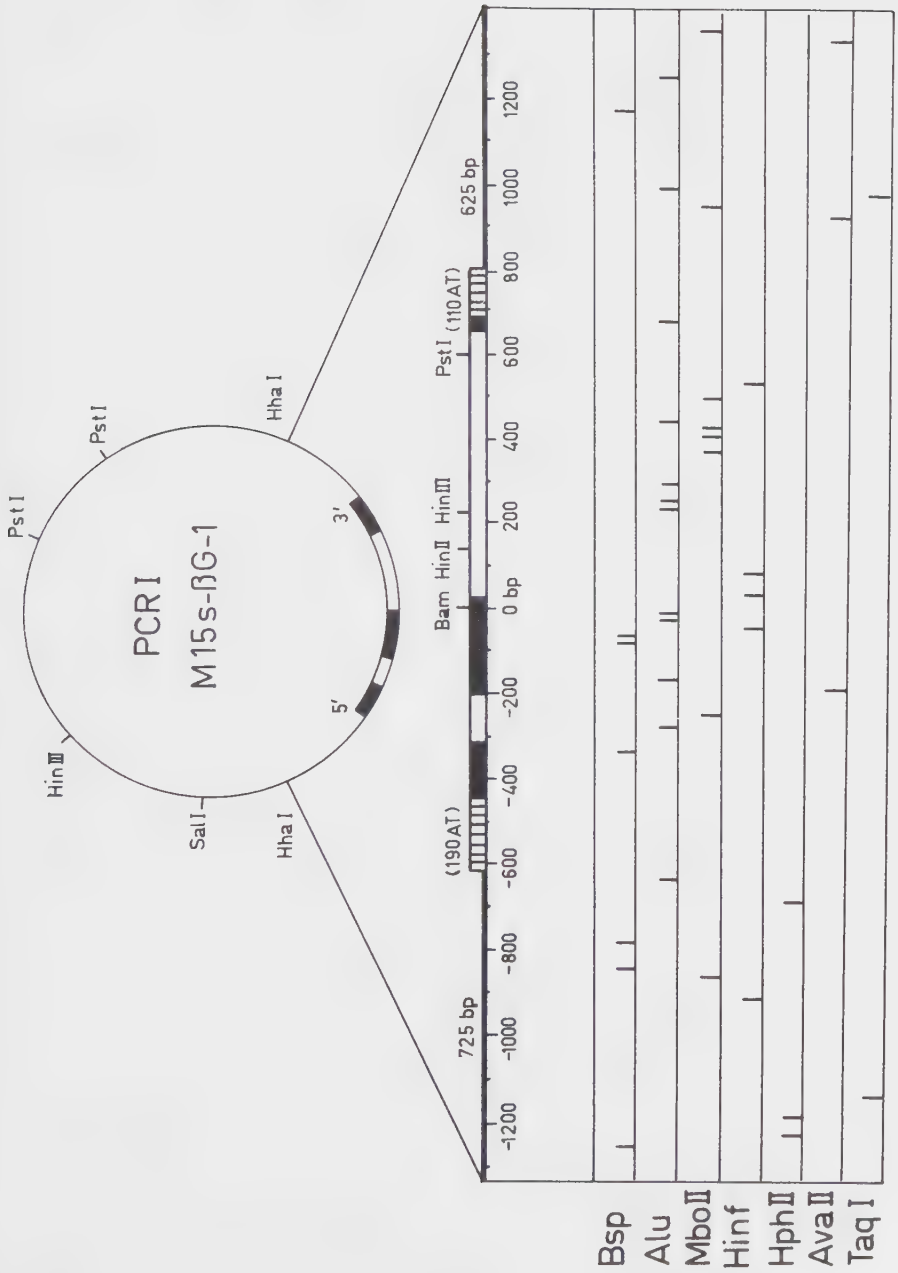
Note: Position one in Figures 11 and 12 corresponds to the position of the 5' cap nucleotide of mRNA. This position corresponds to nucleotide 79 in KONKEL et al. 1978 and to nucleotide 81 in KONKEL et al. 1979.

The data collected on the insert of clone M15s-~~B~~G-1 strongly suggest that the 15s precursor is a true copy of the chromosomal gene containing the small and the large introns, although the 5' and 3' ends were missing on the clone. It is a common experience that the region corresponding to the 5' end of a mRNA is incompletely transcribed, and therefore not represented in the cloned cDNA. The absence of a segment corresponding to the 3' end of the mRNA can be ascribed to excessive degradation during S₁ treatment of the double-stranded cDNA, or to nicking and addition of poly-nucleotide tails at the nicks rather than at the termini.

Figure 9: Restriction map of the clone M15s- β G-1

The DNA was 5' terminally labeled using ^{32}P -gamma ATP and the two fragments obtained after cleavage with HhaI were subjected to partial restriction mapping as described by SMITH & BIRNSTIEL 1976. Similarly the HhaI fragment was labeled and the two fragments obtained after HinIII cleavage were mapped from the other ends. 25 restriction sites were mapped in the insert which were later confirmed by a detailed nucleotide sequence analysis (Figure 11). A part of the final 5' and 3' ends was found to be missing in clone M15s- β G-1, when compared to the known initiation and termination sites in the mouse beta major globin gene (KONKEL et al. 1979, WEAVER & WEISSMANN 1979, see Figure 11). The length of the flanking AT-stretches were estimated by coelectrophoresing marker fragments alongside the sequencing tracks.

Legend:		coding regions
		intervening sequences (introns)
		flanking plasmid sequences
		AT-linkers



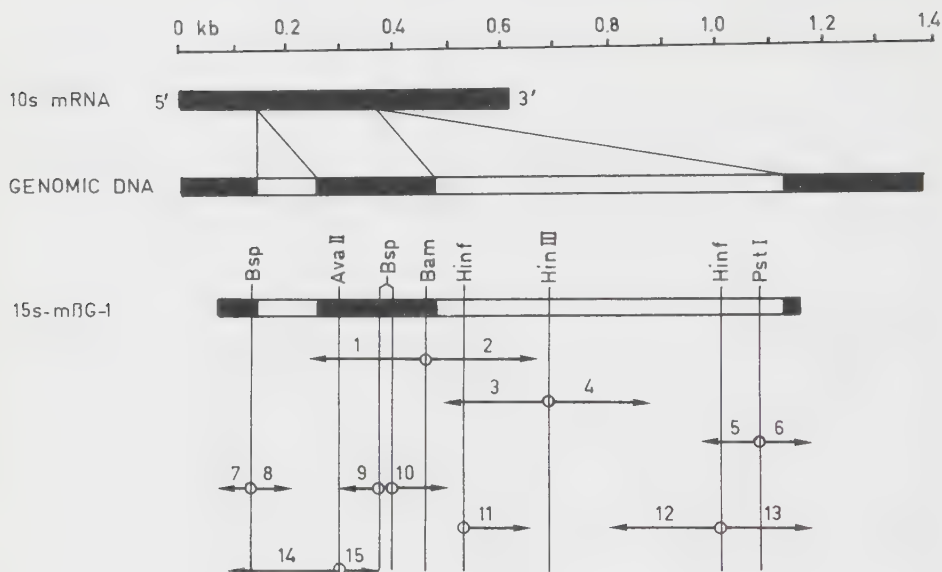


Figure 10: Sequencing strategy for the clone M15s-βG-1, comparison with the cloned chromosomal mouse beta major globin gene (KONKEL et al. 1979)

The DNA was 5' terminally labeled at the indicated sites, starting either with total plasmid DNA or with the 2750 bp HhaI fragment (Figure 9), purified on an 1.5% agarose gel as described in the Materials and Methods section. All fragments are designated with continuous numbers and listed in Table 6. The Figure shows schematically the comparison to the cloned chromosomal mouse beta major gene (KONKEL et al. 1979).

Legend: coding sequences (exons)
 intervening sequences (introns)

Table 6: Fragments prepared for the nucleotide sequence determination of clone M15s- β G-1

starting material	first enzymatic cleavage and labeling	second cleavage	fragments isolated	
total plasmid	BamHI	MboII	1	281 bp
			2	380 bp
total plasmid	HinIII	BspI	3	297 bp
			4	950 bp
total plasmid	PstI	BspI	5	680 bp
			6	580 bp
HhaI fragment	Bsp	Alu	7	280 bp
		MboII	8	58 bp
			9	163 bp
			10	450 bp
HhaI fragment	Hinf	HinIII	11	128 bp
			12	324 bp
		BspI	13	620 bp
HhaI fragment	AvaII	BspI	14	148 bp
			15	72 bp

Figure 11: Reconstructed nucleotide sequence of 15s RNA; clone M15s- β G-1 contains an almost full length cDNA copy of 15s mouse beta major globin precursor RNA.

The sequencing strategy outlined in Figure 10 was followed using the method described by MAXAM & GILBERT 1977. As compared to the chromosomal DNA sequence 52 nucleotides were missing at the 5' end and 233 at the 3' end. They were completed by the known sequence of genomic DNA and underlined (KONKEL et al. 1979). The printed sequence with exception of the underlined part corresponds to clone M15s- β G-1. The nucleotides written in boxes above the printed 15s-sequence represent the sequence of the chromosomal beta globin DNA where it differs from the 15s cDNA sequences and are described in detail in Figure 12. The differences in position 308 and 765 are authentic and not due to sequencing errors (Figure 12). Position one in this Figure corresponds to the 5' cap nucleotide of mRNA which is identical with position 81 in KONKEL et al. 1979. "cap", 5' end of 10s mRNA and 15s precursor RNA (WEAVER & WEISSMANN 1979) "pA", 3' end poly-A addition site

10 20 30 40 50
GCAGGAGCCAGGGCAGAGCATATAAGGTGAGGTAGGATCAGTTGCTCCTC

cap HinII Hinf
0 ACATTTGCTTCTGACATAGTTGTGTTGACTCACAAACCCAGAAACAGACA

50 TCATGGTGCACCTGACTGATGCTGAGAAGGCTGCTGTCTCTTGCCCTGTGG
MetValHisLeuThrAspAlaGluLysAlaAlaValSerCysLeuTrp

Bsp
100 GGAAAGGTGAACTCCGATGAAGTTGGTGGTGAAGCCCTGGGCAGGTTGGT
GlyLysValAsnSerAspGluValGlyGlyGluAlaLeuGlyArg

Alu MboII
150 ATCCAGGTTACAAGGCAGCTCACAAGAAGAAGTTGGGTGCTTGGAGACAG

200 AGGTCTGCTTTCCAGCAGACACTAACTTTTCAGTGTCCCCTGTCTATGTTT

AvaII
250 CCCTTTTTAGGCTGCTGGTTGTCTACCCTTGGACCCAGCGGTACTTTGAT
LeuLeuValValTyrProTrpThrGlnArgTyrPheAsp

Alu G
300 AGCTTTAGAGACCTATCCTCTGCCTCTGCTATCATGGGTAATGCCAAAGT
SerPheArgAspLeuSerSerAlaSerAlaIleMetGlyAsnAlaLysVa

Bsp Bsp Hinf
350 GAAAGCCCATGGCAAGAAGGTGATAACTGCCTTTAACGATGGCCTGAATC
lLysAlaHisGlyLysLysValIleThrAlaPheAsnAspGlyLeuAsnH

Alu
400 ACTTGGACAGCCTCAAGGGCACCTTTGCCAGCCTCAGTGAGCTCCACTGT
isLeuAspSerLeuLysGlyThrPheAlaSerLeuSerGluLeuHisCys

Alu BamHI Hinf
450 GACAAGCTGCATGTGGATCCTGAGAACTTCAGGGTGAGTCTGATGGGCAC
AspLysLeuHisValAspProGluAsnPheArg

500 CTCCTGGGTTTCCTTCCCCTGGCTATTCTGCTCAACCTTCTATCAGAAA

10 20 30 40 50
 ' ' ' ' '
 Hinf
 550 AAAAGGGGAAGCGATTCTAGGGAGCAGTCTCCATGACTGTGTGTGGAGTG

 HinII
 600 TTGACAAGAGTTTCGGATATTTATTCTCTACTCAGAATTGCTGCTCCCCC

 HinIII/Alu
 650 TCACTCTGTTCTGTGTTGTCATTTCTCTTTCTTTGGTAAGCTTTTAATT

 Alu
 700 TCCAGTTGCATTTTACTAAATTAATTAAGCTGGTTATTTACTTCCCATCC

 Alu T
 750 TGATATCAGCTTCCCTCCTCCTT-CCTCCCAGTCCTTCTCTCTCCTC

 TT-CT-CT-CTT-G
 800 TCTCTTTCTCTAATCCTTTCTTTCCCTCAGTTTCATTTCTTCTTTTGA
 MboII
 850 TCTACGTTTGTTTGCTTTTTTAAATATTGCCTTGTAACCTGCTCAGAGGA

 MboII MboII Alu
 900 CAAGGAAGATATGTCCTGTTTCTTCTCATAGCTCTCAAGAATAGTAGCA

 MboII
 950 TAATTGGCTTTTATGCCAGGGTGACAGGGGAAGAAATATTTTACATATA

 Hinf
 1000 AATTCTGTTTGACATAGGATTCTTATAATAATTTGTCAGTAGTTTAAGGT

 PstI
 1050 TGCAAACAAATGCTTTGTAAATAAGCTGCAGTATCTGGTATTTTTGCT

 MboII Alu
 1100 CTACAGTTATGTTGATGGTTCTTCCATATTTCCACAGCTCCTGGGCAATA
 LeuLeuGlyAsnM

	10	20	30	40	50
	'	'	'	'	'
		Bsp			
1150	<u>TGATCGTGATTGTGCTGGGCCACCACTTGGCAAGGATTTACCCCCGCT</u> etIleValIleValLeuGlyHisHisLeuGlyLysAspPheThrProAla				
				Bsp	
1200	<u>GCACAGGCTGCCCTTCCAGAAGGTGGTGGCTGGAGTGGCCACTGCCCTTGGC</u> AlaGlnAlaAlaPheGlnLysValValAlaGlyValAlaThrAlaLeuAl				
1250	<u>TCACAAGTACCACTAAACCCCTTTCTGCTCTTGCCCTGTGAACAATGGT</u> aHisLysTyrHisTer				
1300	<u>TAATTGTTCCCAAGAGAGCATCTGTCAGTTGTTGGCAAAATGATACACAT</u>				
		MboII		pA	
1350	<u>TTGAAAATCTGTCTTCTGACAAATAAAAAGCATTTATGTTCACTGCAATG</u>				

Figure 12: Nucleotide sequence differences between the M15s- β G-1 cDNA and the beta major globin genomic DNA

The sites of nucleotide changes were photographed from the original autoradiograms showing the specific degradation products of the G, A, T and C reactions according to the protocol of MAXAM & GILBERT 1977. The indicated positions correspond to the nomenclature used in Figure 11.

Panel A shows the sequence of the clone M15s- β G-1 at the position 308 (indicated in Figure 11). The A at the indicated site is clearly visible.

Panel B shows the same sequence read from the opposite strand of genomic DNA as determined by N. MANTEI. C is read at position 308 (Figure 11) corresponding to a G (and not to an A) on the complementary strand.

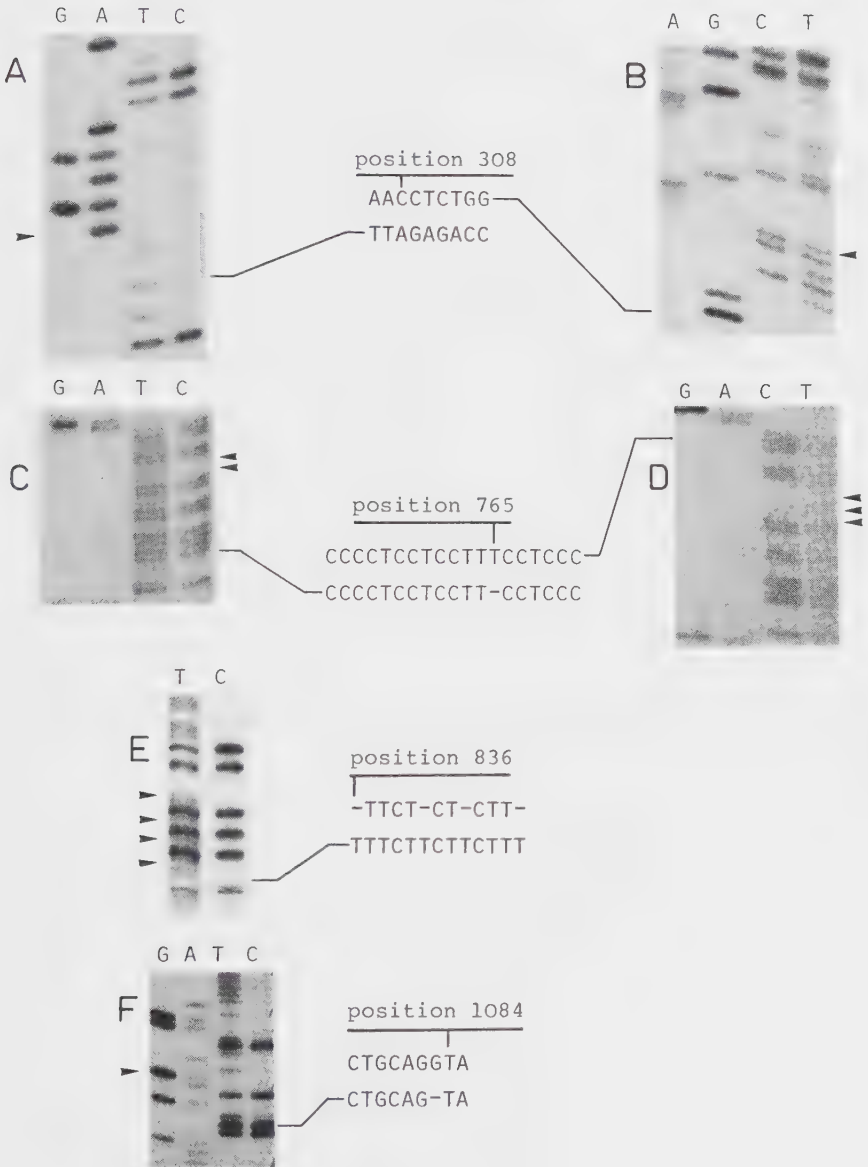
Panel C and D show the homologous region in position 765 (indicated in Figure 11). The autoradiograms were of low quality and the difference in the number of T's is best estimated from the gaps in the C-lanes.

Panel E shows the situation at position 836 of the clone M15s- β G-1 (indicated in Figure 11). The T's indicated by arrows are close to the C bands and can be missed on a low quality gel. Although the original gels of the chromosomal DNA (not shown) were not satisfying in this region the sequence determination was not repeated.

Panel F shows position 1084 following the PstI site of clone M15s- β G-1. Clearly no second G can be detected. This sequence is in agreement with the chromosomal sequence determined by N. MANTEI (Van OUYEN et al. 1979) but not with the sequence published by KONKEL et al. 1979.

M15s- β G-1

GENOMIC DNA



Analysis of the insert of clone M15s- β G-3, a cDNA representing a possible splicing intermediate.

The plasmid DNA M15s- β G-3 was cleaved and 5' terminally labeled at the unique BamHI site. After cleavage with TaqI two fragments of about 500 and 900 bp were isolated and subjected to partial restriction mapping as described by SMITH & BIRNSTIEL 1976. The 900 bp fragment had a restriction map typical for the large intron, as shown in Figure 13. The 500 bp fragment showed the sites typical for the 5' coding portion, however the distance between two Bsp sites, which span the small intron in genomic DNA (KONKEL et al. 1979) was about 105 bp smaller than expected, suggesting the absence of the small intron. This structure might represent an intermediate splice product containing no small intron but at least part of the large intron. The S_1 -mapping experiment described in Scheme 4 and Figure 17 showed that the clone M15s- β G-3 was colinear with chromosomal DNA from the BamHI site upstream to the 3'-border of the small intron (205 bp) and therefore lacked the small intron; and was colinear with beta globin chromosomal DNA from the BamHI site to a position 500 bp downstream (and therefore contains 485 bp of the large intron). In addition, clone M15s- β G-3 was partially sequenced, as shown in Figure 14. It extends from the beginning of the 5'-coding region, across the splice point of the small intron, (which was absent) into the middle exon and follows into the large intron. The sequenced part was identical with the beta major globin gene (KONKEL et al. 1979). The base difference at position 308 (Figure 11) between clone M15s- β G-1 and chromosomal beta globin DNA was not detected in this clone.

Figure 13: Comparison of the restriction maps of clone M15s- β G-3 and cloned beta major globin chromosomal DNA.

The DNA of M15s- β G-3 was 5' terminally labeled at the BamHI site using 32 P-gamma ATP and cleaved with TaqI. The two resulting labeled fragments of 500 and 900 bp were subjected to partial restriction mapping (SMITH & BIRNSTIEL 1976) with the enzymes Bsp, Alu and MboII. The regions indicated by arrows on the bottom of the Figure were used for the partial sequence analysis shown in Figure 14.

Legend:  coding sequences (exons)
 intervening sequences (introns)
 AT-linkers
 flanking plasmid sequences

chromosomal beta major globin DNA

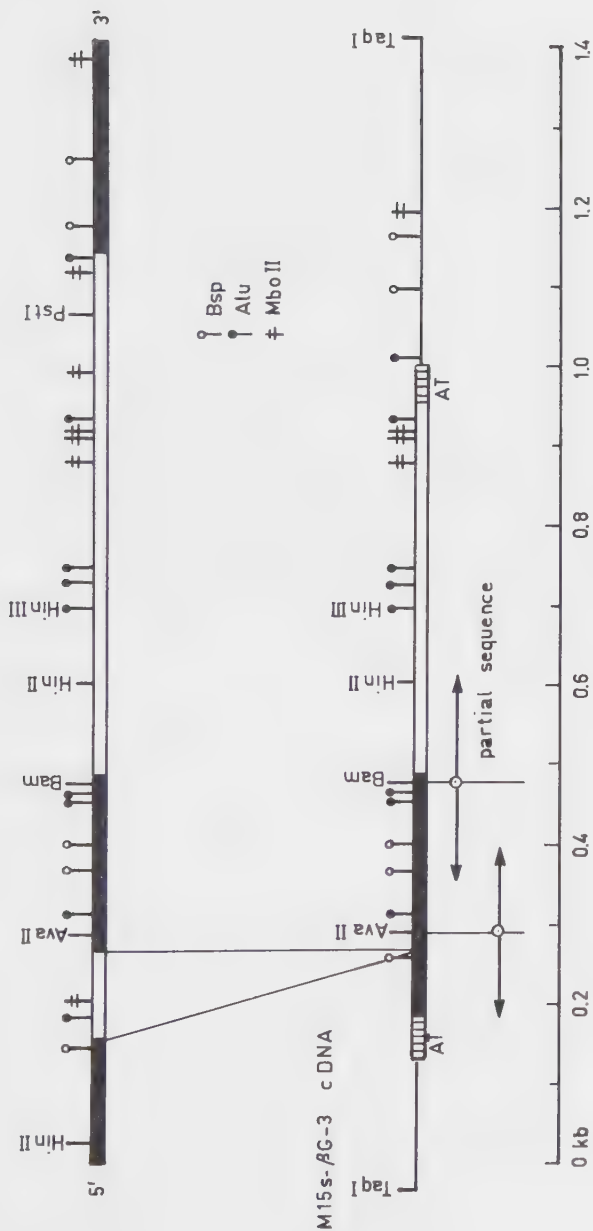


Figure 14: Partial nucleotide sequence analysis
of clone M15s- β G-3

The DNA of M15s- β G-3 was digested with *Ava*II or *Bam*HI, 5' terminally labeled using 32 P-gamma ATP and sequenced into both directions (Figure 13) following the method described by MAXAM & GILBERT 1977. The first nucleotides were deduced from the known recognition sequences of the restriction sites.

The position of the splice point of the small intron is indicated by a triangle. 51 nucleotides were missing from the cap position of the 5' end. The segment corresponding to the large intron was not sequenced to the end. By S_1 -mapping (see Figure 17) it was shown, that 485 nucleotides complementary to genomic beta major globin from the *Bam*HI site into the large intron were present.

	10	20	30	40	50

51 - ATCATGGTGCACCTGACTGATGCTGAGAAGGCTGCTGTCTCTTGCCTGTG
MetValHisLeuThrAspAlaGluLysAlaAlaValSerCysLeuTr

splice point


101 - GGGAAAGGTGAACTCCGATGAAGTTGGTGGTGAGGCCCTGGGCAAGGCTGC
pGlyLysValAsnSerAspGluValGlyGlyGluAlaLeuGlyArgLeuL

AvaII

151 - TGGTTGTCTACCCTTGGACCCAGCGGTACTTTGATAGCTTTGGAGACCTA
euValValTyrProTrpThrGlnArgTyrPheAspSerPheGlyAspLeu

201 - TCCTCTGCCTCTGCTATCATGGGTAATGCCAAAGTGAAGGCCCATGGCAA
SerSerAlaSerAlaIleMetGlyAsnAlaLysValLysAlaHisGlyLy

251 - GAAGGTGATAACTGCCTTTAACGATGGCCTGAATCACTTGGACAGCCTCA
sLysValIleThrAlaPheAsnAspGlyLeuAsnHisLeuAspSerLeuL

301 - AGGGCACCTTTGCCAGCCTCAGTGAGCTCCACTGTGACAAGCTGCATGTG
ysGlyThrPheAlaSerLeuSerGluLeuHisCysAspLysLeuHisVal

BamHI

351 - GATCCTGAGAACTTCAGGGTGAGTCTGATGGGCACCTCCTGGGTTTCCTT
AspProGluAsnPheArg

401 - CCCCTGGCTATTCTGCTCAACCTTCCTATCAGAAAAAAGGGGAAGCGAT

451 - TCTAGGGAGCAGTCTCCATGACTGTGTGTGGAGTGTTGACAAGAGTTCGG

501 - ATATTTTATTCTCTACTCAGAATTGCTGCTCCCCCTCACTCTGTTCTGTG

551 - TTGTCATTTCTCTTTCTTTGGTAAGCTTTTAATTTCCAGTTGCATTTTA

Analysis of clone M15s- β G-6, comparison with the cloned chromosomal DNA of a beta minor globin gene.

The plasmid DNA M15s- β G-6 was cleaved with BamHI, 5' terminally labeled with 32 P-phosphate and digested with HinIII. The two fragments of 4500 bp and 9500 bp respectively, were isolated and subjected to partial restriction mapping (SMITH & BIRNSTIEL 1976). The 4500 bp fragment showed the Alu and Bsp sites typical for the 5' coding portion of the beta globin gene lacking the small intron. By DNA sequencing 8 nucleotide changes characteristic for the beta minor globin gene were detected (Figure 16). The 3' fragment showed all the restriction sites typical for the large intron of a beta minor globin gene and part of the 3' end coding sequence, but again no complete 3' end was present. (The second, smaller HhaI fragment which was observed in Figure 8 and was present in non-stoichiometric amounts, must be considered as an artefact or a contaminant. The restriction mapping data are summarised in Figure 15 and a partial nucleotide sequence analysis is shown in Figure 16.

These data show that M15s- β G-6 is derived from a beta minor globin transcript which contains the large but not the small intron, as was the case for clone M15s- β G-3 (Figures 13 and 14).

Figure 15: Comparison of the restriction maps of clone M15s-~~B~~G-6 and of the cloned chromosomal beta major and minor globin genes.

The plasmid DNA M15s-~~B~~G-6 was 5' terminally labeled at the BamHI site using ³²P-gamma ATP and the two fragments obtained after HinIII cleavage were subjected to partial restriction mapping (SMITH & BIRNSTIEL 1976). For comparison the structures of the completely sequenced beta major and minor globin genes (KONKEL et al. 1979) are drawn schematically above the 15s sequence. The arrows at the bottom of the map indicate the partially sequenced regions (Figure 16).

Legend:  coding sequences (exons)
 intervening sequences (introns)
 AT-linkers
 flanking plasmid sequences

chromosomal beta major globin DNA



chromosomal beta minor globin DNA

M15s- β G-6 cDNA

	10 ↓	20 ↓	30 ↓	40 ↓	50 ↓
	<u>AvaII</u>		<u>Alu</u>		
473 -	CCCTTGGACCCAGCGGTACTTTAGATAGCTTTGGAGACCTATCCTCTGCC				
		Ⓜ	Ⓜ	<u>Bsp</u>	Ⓜ
523 -	TCTGCTATCATGGGATATCCCAAGGTGAAGGCCCATGGCAAAAAGGTGAT				
		Ⓜ	<u>Bsp</u>	Ⓜ	Ⓜ
573 -	AACTGCCTTTAACGAGGGCCTGAAAAACCTGGACAACCTCAAGGGCACCT				
		Ⓜ	<u>Bsp</u>	Ⓜ	Ⓜ
			<u>Alu</u>	<u>Alu</u>	<u>BamHI</u>
623 -	TTGCCAGCCTCAGTTGAGCTCCACTGTGACAAGCTGCATGTGGATCCTGA				
			<u>Alu</u>	<u>Alu</u>	<u>BamHI</u>
		<u>Hinf</u>			
673 -	GAACTTCAGGGTGAGTCTGATGGGCACCTCCTGGGTTTCCTTCCCCTGGC				
723 -	TATTCTGCTCAACCTTCCTATCAG				

Figure 16: Partial nucleotide sequence determination of the clone M15s- β G-6, identification as a beta minor globin type.

The plasmid DNA was 5' terminally labeled at the unique BamHI site and subjected to nucleotide sequence analysis as described by MAXAM & GILBERT 1977 into both directions. 8 base changes in the 5' coding part characteristic for a beta minor globin gene (KONKEL et al. 1979) were identified. The corresponding nucleotides of the beta major globin gene are drawn in circles above the sequences of clone M15s- β G-6. The first 60 nucleotides from the BamHI site into the large intron do not show differences between major and minor beta globin. The region of the sequenced part is indicated by arrows on the bottom of Figure 15.

Characterization of the clones M15s- β G-3 and M15s- β G-6 by S_1 -mapping.

An S_1 assay similar to that developed by BERK & SHARP 1977 and WEAVER & WEISSMANN 1979 to map RNA molecules on their DNA counterparts was used to characterize the 15s clones. Cloned chromosomal beta major globin DNA (LEDER et al. 1977) was cleaved with BamHI, labeled with 32 P-phosphate, digested with EcoRI and the two labeled fragments were purified. The 15s plasmid DNAs were linearized by HinIII or HhaI cleavage, mixed with the probe, denatured and hybridized. The hybridized DNAs were successively treated with the single strand specific nuclease S_1 and the protected fragments were run on 5% polyacrylamide gels containing 7 M urea as described by WEAVER & WEISSMANN 1979 and in the Methods section.

The principle of the assay is drawn schematically in Scheme 4. The 15s cDNAs protected predominantly the fragments expected from partial restriction mapping and confirmed the structures drawn in Figure 18 (results not shown). Figure 17 shows as an example the results of an assay of the clones M15s- β G-3 (beta major globin splicing intermediate, see Figure 13) and M15s- β G-6 (beta minor globin splicing intermediate, see Figure 15) compared with the almost full length clone M15s- β G-1 (see Figure 11) and a 10s-cDNA clone pCRI/m β 1. The structures presented in the Figures 13 and 15 were confirmed by the results shown in Figure 17 and explained in Scheme 4. The 5' portion is interrupted in both clones, M15s- β G-3 and 6, at the same position as the 10s cDNA clone, indicating the looping out of the small intron in the labeled genomic DNA at the position where the small intron was absent in the 15s clones. The 3' portion protects a fragment of 500 bp for the clone M15s- β G-3 indicating strong homology with a large portion of the adjacent large intron sequence of the beta major probe. Clone M15s- β G-6 protects a fragment of only 110 bp, because of the sequence divergence between the beta major and minor

genes as determined by KONKEL et al. 1979. The renatured probe contains labeled 5' overhanging ends and is usually completely degraded by S_1 nuclease. In most assays a faint band corresponding to renatured probe is visible at the indicated positions in Figure 17.

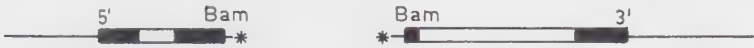
Scheme 4: Principle of heteroduplex formation and S_1 -mapping of 15s cDNA clones.

A: The probes were prepared by labeling cloned plasmid DNA (chromosomal beta major globin, pBR/LEM) at the BamHI sites. The 5' (2.1 kb) fragment and the 3' (5 kb) fragment of beta major globin DNA were purified separately after EcoRI cleavage by agarose gel electrophoresis as described in Materials and Methods. The designations 5' and 3' in all the fragments and heteroduplex molecules correspond to the 5' and 3' ends of mature 10s mRNA. In order to make the formation of the major products in this assay better understandable, the complementary DNA strand (unlabeled), which is always present for each fragment is not drawn in this scheme. The formation of labeled heteroduplex molecules was favoured by the addition of a large excess of cold DNA over labeled probe DNA. It should be noted that in addition to heteroduplexes, homoduplexes and even networks may be formed. Some of the minor bands which are sometimes visible in this type of assay may originate from such structures, although S_1 nuclease should degrade all side products.

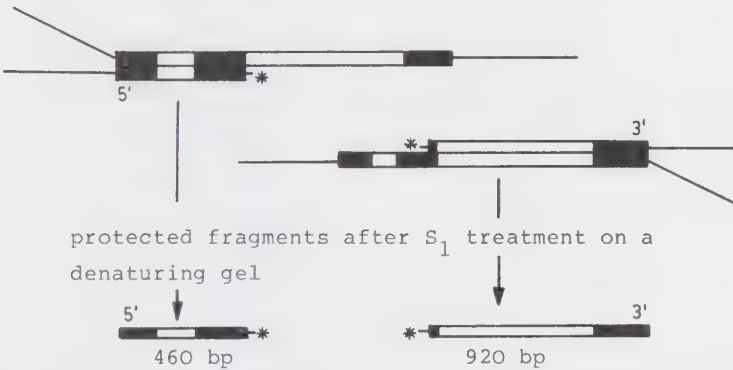
B: The formation of a heteroduplex molecule between denatured probes (described under A) and a denatured full length 15s beta major globin cDNA copy is drawn schematically. All the single-stranded regions are then cleaved by the single-strand specific nuclease S_1 . The labeled protected fragment can be sized after denaturation by acrylamide gel electrophoresis as described in the Methods section.

C: The formation of heteroduplex molecules between denatured probes (described under A) and denatured full length 10s beta major globin cDNA is drawn schematically. Loop structures are formed at the positions of nonhomologies where the introns start, and are cleaved by S_1 nuclease. The labeled and protected fragment only extends to the beginning of the loop and can be detected after denaturation by acrylamide gel electrophoresis.

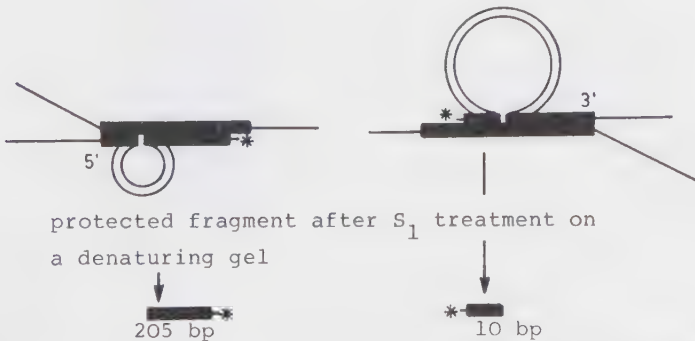
A Bam 5' end labeled probes representing cloned chromosomal beta major globin DNA



B Hybridization of linearized full length 15S cDNA to the 5' and 3' labeled probes, respectively



C Hybridization of full length 10S cDNA to the 5' and 3' labeled probes, respectively



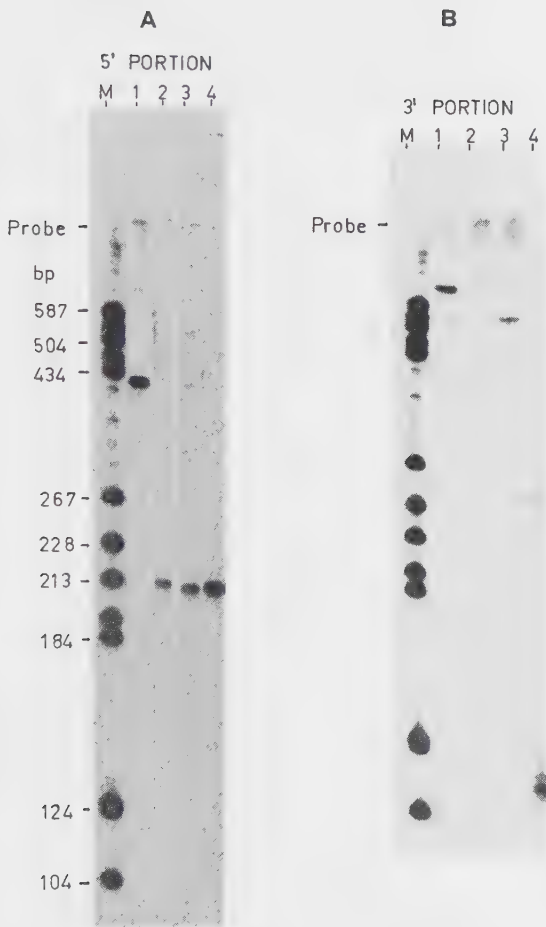
Legend:

- regions corresponding to mature 10S mRNA
- intervening regions (introns)
- nonhomologous flanking regions
- 5' ³²P-labeled ends

Figure 17: S_1 -mapping of the clones M15s- β G-3
and M15s- β G-6

The principle of the assay is explained in Scheme 4. 5000 cpm of denatured probe (specific activity 10^6 cpm per pMol of ends) were hybridized to 1 μ g of denatured 15s cDNA. The hybrids were successively treated with S_1 nuclease and the protected labeled fragments were analysed on a 5% acrylamide gel containing 7 M urea in 50 mM tris-borate buffer as described in detail in the Methods section.

Autoradiogram A shows the protected fragments obtained with the labeled probe towards the 5' end, autoradiogram B shows the fragments towards the 3' end of the gene. M are marker fragments, pBR322 plasmid DNA cleaved with Bsp. Slot number one shows the protected fragments obtained with the almost full length 15s cDNA clone M15s- β G-1, of 420 bp (5') and 680 bp (3'), as confirmed by the sequencing data Figure 11. Slot number 2 shows the control fragments obtained with the 10s beta major globin cDNA clone (m β 1). A fragment of 205 bp was protected towards the 5' end as explained in Scheme 4 example C. The 10 bp fragment in the 3' portion run off the gel. Slots number 3 and 4 (clones M15s- β G-3, beta major splicing intermediate, see Figure 13, and clone M15s- β G-6, beta minor splicing intermediate, see Figure 15) show the same protected fragment in the 5' portion as the 10s cDNA clone. The 3' end shows homology of 500 bp for clone M15s- β G-3 and of 110 bp for clone M15s- β G-6 (explanations in the text).



Summary of the collected data on the 15s-cDNA clones.

Within the framework of this thesis it was not possible to elucidate the structures of all the inserts of the 15s clones by nucleotide sequence analysis. A combination of methods was used to establish the structures presented schematically in Figure 18. The clones were analysed as follows:

1. By colony hybridization to a specific fragment representing the large intron sequence of a cloned beta major globin gene (Figure 7).
2. By estimating the size of the largest fragment of an HhaI digest containing the inserted 15s cDNA by agarose gel electrophoresis (Figure 8).
3. All the clones contained a unique BamHI site, which could be 5' terminally labeled, cleaved with a second restriction enzyme and the two resulting labeled fragments could be mapped by partial cleavages with various restriction enzymes. The DNAs of the clones number 10 and 11 were of low quality and could not be labeled. An example of this assay is described for clone M15s- β G-1 in Figure 9.
4. By a complete nucleotide sequence analysis of the insert of clone M15s- β G-1 (Figure 11, beta major globin); by a partial nucleotide sequence analysis of the clones M15s- β G-3 (Figure 14, beta major globin, splicing intermediate), and M15s- β G-6 (Figure 16, beta minor globin, splicing intermediate), and M15s- β G-7 which contained 10s sequences only (data not shown).
5. By an S₁-mapping assay (BERK & SHARP 1977, WEAVER & WEISSMANN 1979). An example is shown in Figure 17.

The frequencies of the different beta globin precursor species in this cloning experiment are drawn in Table 7. The number of clones examined is very small and the percentage therefore not very meaningful. The 10s cDNA clone number 7 was eliminated for this estimation.

Table 7: The relative frequencies of globin precursor species

type	number of clones	frequency
total	11	100 %
major	8	72.5 %
minor	3	27.5 %
major, splicing intermediate	1	9.0 %
minor, splicing intermediates	3	27.5 %

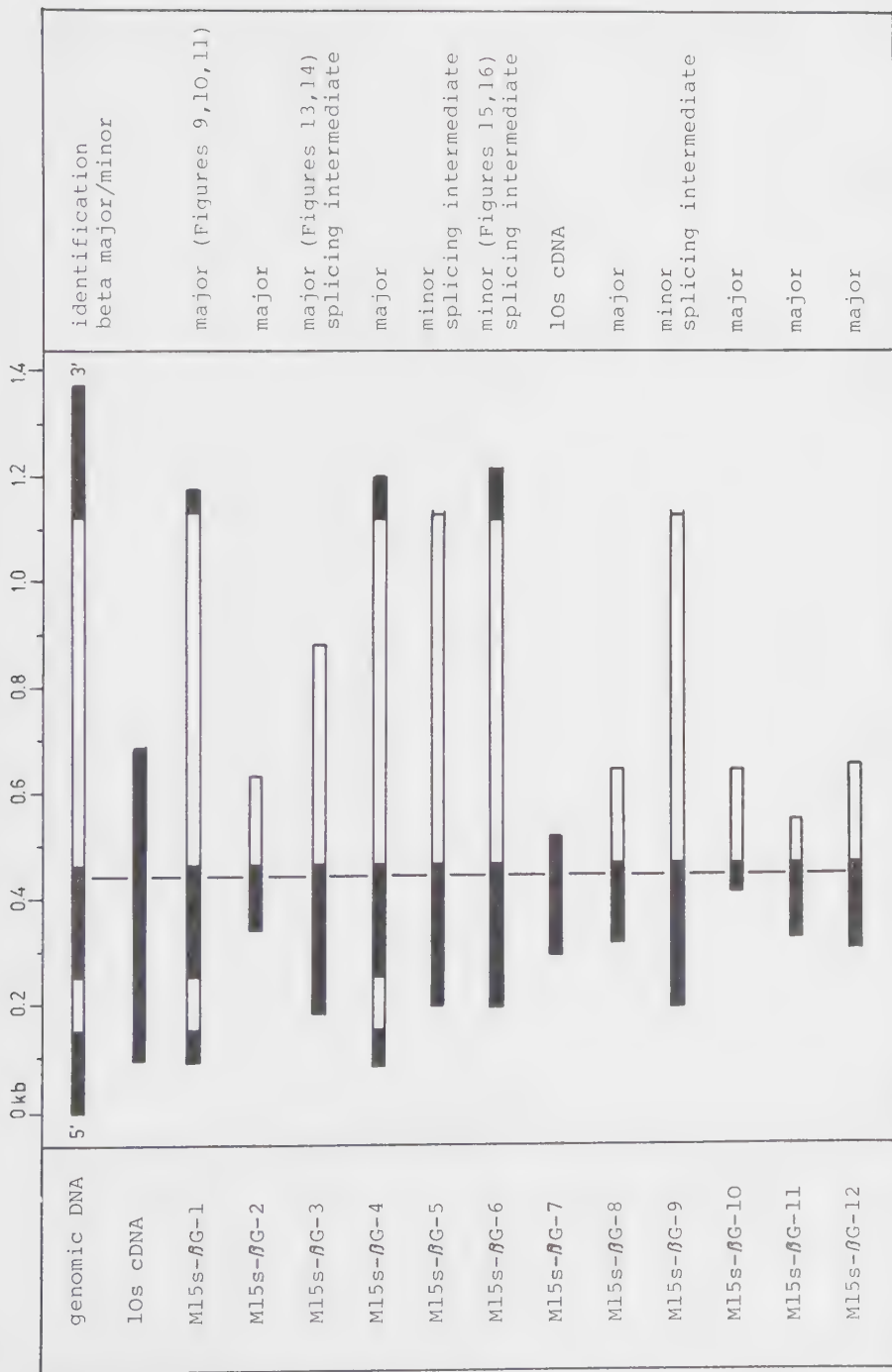
The ratio of major to minor beta globin was estimated to 80% and 20% on the protein level (BARRY WHITNEY III 1977). The ratio found at the RNA level reflects this value quite well.

Figure 18: Structures of the 15s cDNA clones derived from the collected data.

The clones were characterised as summarised in the text, and the structures drawn schematically. For comparison, the structures of genomic beta globin DNA and 10s beta globin cDNA (corresponding to mature mRNA) were drawn at the top of the Figure. 5' and 3' ends are indicated.

The identification as major or minor beta globin was based on either sequencing, or the presence of a TaqI restriction site specific for the minor species, 110 bp from the left edge of the large intron (KONKEL et al. 1979, see Figure 15), or by the fragments protected in an S_1 -mapping experiment due to mismatches within the large intron between the beta major globin probe and the beta minor globin DNA. The splicing intermediates were identified by the Bsp restriction sites in the 5' portion (Figures 13, 15), or by the fragments protected in an S_1 -mapping experiment (Figure 17).

Legend:  coding sequences (exons)
  intervening sequences (introns)



DISCUSSION:

The rabbit beta globin gene

A 5.1 kb KpnI fragment of rabbit chromosomal DNA containing the beta globin gene was cloned using a technique that is generally applicable to unique eukaryotic genes. Its structure was determined and compared (Van Ooyen et al. 1979) to the mouse beta globin gene cloned in a lambda vector and characterised by KONKEL et al. 1978, as well as to a restriction map of chromosomal rabbit DNA determined by JEFFREYS & FLAVELL 1977. The comparison showed very convincingly that these genes are split by sequences not found in mature mRNA. In fact, most eukaryotic genes which have since been investigated in detail follow a similar structural arrangement of coding parts interrupted by intervening sequences or introns, an organization now recognized as a general phenomenon in eukaryotic structural genes.

The cloned genes, in either a bacterial plasmid or in a lambda phage vector are exposed to the replication machinery of the host. Despite the fact that the host strains used are $recA^-$ and therefore defective in at least some recombinational functions, cloned DNA may contain molecular rearrangements. HARDISON et al. 1979 cloned a chromosomal rabbit DNA fragment in a lambda vector and identified four closely linked beta-like globin genes. The gene which is located closest to the 3' end, designated β_1 (HARDISON et al. 1979), codes for the beta globin chain in adult animals. It was completely sequenced (HARDISON et al. 1979) and corresponds to the gene cloned and sequenced earlier in the 5.1 kb KpnI fragment by us (Van Ooyen et al. 1979). A comparison of these two independently determined nucleotide sequences indicates the fidelity of the cloning procedure and the replication within the host. The coding region of the rabbit beta globin cDNA clone p β G (MANIATIS et al. 1977) agreed with the coding parts of the cloned genomic KpnI fragment analysed in our laboratory.

HARDISON et al. 1979 localised 4 characteristic base differences in the coding sequence and two in the large intron of the gene β_1 by comparison with our sequence (Van Ooyen et al. 1979). GALLIZI 1970, 1971, SHAMSUDDING et al. 1973 and BRICKER et al. 1974 described allelic rabbit beta globin peptides and HARDISON et al. 1979 were able to correlate the β_1 -gene with a codominant rabbit beta globin allele described by these authors. The nucleotide and amino acid changes correspond to the following positions in Figure 6: 336 (A $\rightarrow$ C, Asn $\rightarrow$ His), 349 (A $\rightarrow$ G, Asn $\rightarrow$ Ser), 409 (G $\rightarrow$ A, Ser $\rightarrow$ Asn), 1089 (A $\rightarrow$ G, Ile $\rightarrow$ Val). The changes in the large intervening sequence correspond to positions 937 (T $\rightarrow$ C) and 1057 (T $\rightarrow$ C). Apart from these changes the two reported sequences are in complete agreement. The existence of two allelic rabbit beta globin genes was confirmed by the peptide analysis, and point to the fact of genetic polymorphism.

So far no artificial nucleotide changes occurring during the cloning and propagation of natural DNA have been reported. However LACY et al. 1979 described an artificial deletion of 5 kb in a lambda recombinant phage within a repeat sequence close to the rabbit beta globin gene β_1 . Similar observations were made in Charon 4A recombinant phages containing the human alpha (J. LAUER) and gamma (E. FRITSCH) genes as mentioned by LACY et al. 1979.

Cloned material can provide faithful genetic information as shown by the expression of in vitro transcribed and cloned $Q\beta$ - phage. Infective $Q\beta$ -particles could be obtained from a recombinant plasmid containing full-length $Q\beta$ -sequences (TANIGUCHI et al. 1978).

In the case of recombinant plasmids containing polyoma sequences, only the structures containing a head to tail dimeric polyoma DNA showed biological activity as monitored by virus production after infection of mouse fibroblasts (FRIED et al. 1979).

The lambda cloning system developed by MANIATIS et al. 1978

is the one that is used most frequently at present. The advantages over the system used in this work are the following: 50 to 100 times less cell DNA is required to represent a whole eukaryotic genome, the DNA content of one individual animal or even one of its organ tissues can be sufficient for one shotgun experiment; the lambda hybrid phages can easily be amplified and stored in aliquots in the form of a phage lysate; and the plaques in filter hybridization assays for plaque screening can be plated at a 10 to 20 fold higher density than in comparable colony filter assays (GRUNSTEIN & HOGNESS 1975).

The molecular structure of mouse 15s beta globin precursor RNA:

Purified 15s beta globin precursor RNA was converted into double-stranded DNAs and cloned in a plasmid vector following the method described by MANIATIS et al. 1976. The nucleotide sequence of the cDNA insert of one of the clones, M15s- β G-1 was completely determined. With 1106 of the 1390 nucleotides of the beta globin major gene it represented an almost full length primary transcript of the beta globin gene. The sequences of the small and the large introns were shown to be present in this transcript. This result was consistent with R-loop mapping experiments between cloned chromosomal DNA and 15s RNA (TILGHMAN et al. 1978). These data demonstrate very convincingly that during globin gene expression a primary transcript which is an exact copy of the chromosomal sequence is formed. The 3' and 5' ends corresponding to mature 10s mRNA were missing in this and in all the other clones, either because the chains were prematurely terminated in the reverse transcriptase reaction, or because the cDNAs were trimmed at the ends by the action of an exonuclease in one of the in vitro steps. It was shown by an S₁-mapping experiment (WEAVER & WEISSMANN 1979) that the 5' ends of 15s precursor and mature 10s mRNA map at the same position on the genomic DNA. The identity of the final 3' ends was not yet experimentally proven. Out of 1106 nucleotides 1104

were identical with the sequence of a cloned chromosomal beta globin major gene (KONKEL et al. 1979).

Two base changes were found: one in the coding sequence (position 308, Figure 11), leading to a codon change Gly to Arg and a second change in the large intron (position 765, Figure 11). How can these changes be interpreted? A number of closely related beta globin chains in different mouse strains have been reported, but no allelic globin variant has this amino acid change (POPP & BAILIFF 1973, WHITNEY 1977, GILMAN 1976, WEAVER 1979b). Amino acid differences are described for beta d major (identical to beta p major), beta d minor, beta p minor and beta s. The data are summarised in Table 8. The sequence determined for clone M15s- β G-1 corresponds to the type beta d major, except for an arginine residue at position 46 (nucleotide position 308, Figure 11). The complex genetic locus expressing beta globin sequences is designated Hbb. Type Hbb^d produces the two proteins beta d minor and major, strains of this type carry the following designations: SWR, NB, NZB/BiM_J, Balb/c. The strain C57BL is of type Hbb^s producing one single type, beta s globin. The strain AU/SsJ is of the type Hbb^p and produces two different beta globin chains, beta p major and minor of which p major is identical with d major and p minor is different from d minor. All these data do not explain the base change in the coding sequence of clone M15s- β G-1. Each cDNA clone represents essentially one single transcript. The error rate of the eukaryotic transcriptional apparatus and of the reverse transcriptase system added up and is difficult to estimate. A second clone M15s- β G-3 which was sequenced at position 308 (Figure 14) did not show this base change; it contained the sequence expected for a beta d major globin gene.

The base changes in clone M15s- β G-1 can be (a) due to nucleotides missincorporated by the in vitro procedures (artifacts) or (b) it represents a transcript of an allele not yet identified. (c) Friend cells are derived from a cloned cell line and went

through many passages without any selective pressure on the globin genes. Accumulation of mutations in the Friend cell genome can not be excluded.

Table 8: Amino acid differences in closely related mouse beta globin chains

Amino acid position	beta d major beta p major	beta d minor	beta p minor	beta s
9	Ala	Ser	Ser	Ala
13	Cys	Cys	Cys	Gly
16	Gly	Ala	Ala	Gly
20	Ser	Pro	Pro	Ala
22	Glu	Glu	Ala	Glu
23	Val	Val	Ile	Val
46	Gly *)Arg			
58	Ala	Pro	Pro	Ala
72	Asp	Glu	Glu	Asp
73	Asp	Glu	Lys	His
76	Asn	Lys	Lys	His
77	His	Asn		
80	Ser	Asn		
109	Met	Ala		
139	Thr	Thr	Thr	Ala

*) The nucleotide sequence data of the 15s-clones correspond to the genotype Hbb^d producing a beta d major and d minor peptide, with exception of the base change at position 308 in clone M15s-~~B~~G-1 which codes for Arg instead of Gly at position 46.

Splicing intermediates of the mouse 15s precursor RNA:

The analysis of further cDNA clones showed an interesting variation in the basic structure of some of the clones (see Figure 13 and 15). Sequences of the large but not of the small intron were present. Again these cDNAs had no complete 3' and 5' ends but the data strongly suggested that they were reverse transcripts of an intermediate splice product containing the large intron and the 5' portion identical to mature 10s mRNA. The same structures were found for both beta major and minor transcripts. (The probe representing the large intron of the beta globin major gene used in colony screening was homologous enough to the large intron of the beta minor gene to pick up the transcripts of this gene as well.) The presence of these cDNAs indicates that splicing of globin 15s precursor RNA is not a one-step reaction. The fact that no structures containing the small but not the large intron were found does not mean that they do not exist, because the way the clones were screened (using a hybridization probe specific for the large intron) excluded such structures. (A probe which was prepared to hybridize to the small intron did not work specifically). It is also not possible to say whether 15s precursor RNA is spliced in any particular order. The process of splicing must be almost as fast as the rate of synthesis of 15s precursor, because intermediates were detected with a frequency similar to that of full length transcripts. No evidence was found for structures containing only parts of the introns flanked by coding sequences as proposed by KINNIBURGH et al. 1979. A different approach to the mapping of primary transcripts and splicing intermediates of the rabbit beta globin gene was undertaken by GROSVELD et al. 1980 (in press). They used a modification of the BERK-SHARP assay (BERK & SHARP 1977, WEAVER & WEISSMANN 1979) and hybridized probes (5' end labeled at specific sites within the introns) to total poly-A RNA, and sized the protected fragments after S_1 nuclease treatment by gel electrophoresis. The protected fragments

indicated the presence of full length 15s rabbit beta globin transcripts and in addition splicing intermediates containing (a) a part of the small and the complete large intron, (b) no small but the complete large intron (similar as shown for clone M15s-~~9~~G-3, Figure 13 in this work), and (c) no small intron but a small part of the large intron.

When individual cloned transcripts of the closely related genes beta major and minor globin were analysed in this work by S_1 -mapping (Figure 17), using the specific hybridization probe of the beta major gene, fragments were found which corresponded to mismatches between the large introns of the two species. Therefore a certain danger of misinterpretation may exist if poly-A RNA is screened for transcripts by S_1 -mapping using a gene specific probe: The specific probe will hybridize to closely related nucleic acid species and S_1 nuclease will cleave the probe at the first region of mismatch. The protected fragment will give the impression of a shorter transcript while it was in fact partially protected by the product of a different gene with a certain region of homology to the labeled end of the probe.

The splicing mechanism and its biological meaning are currently being investigated in many laboratories. Some important observations can be summarised as follows: HAMER et al. 1979 showed that the large intron of the mouse beta globin gene alone was correctly spliced out in a monkey cell line when joined to an SV40 late gene in the proper 5'3' orientation. No splicing occurred when it was inserted in the other orientation. No stable RNA could be detected after removal of the intron sequences from the late SV40 gene - but stable RNA was found if the SV40 intron was replaced by the large intron of the mouse beta globin gene. HAMER & LEDER 1979 postulated that a gene without introns would not be able to produce stable RNA. In contradiction to this statement are the observations made on the human leucocyte interferon system. The chromosomal genes

do not contain introns (NAGATA et al. 1980), but mRNA can be detected. Another example are the histone genes (SCHAFFNER et al. 1978).

The "CHAMBON RULE" (BREATHNACH et al. 1978), which states that the 5' border of an intron starts with GT and the 3' border ends with AG, holds for all introns studied in eukaryotic genes so far.

As viewed on paper most introns could be spliced in several ways, with the splice point shifted one by one nucleotide and still yield the same coding information because of repeats along the borders (Van den BERG et al. 1978). No unique splice point has been exactly determined so far. Technically, the spliced-out fragment would have to be isolated and analysed - and this is not a simple project.

Currently experiments are in progress to study the requirements of "splicing signals". Arteficiel chromosomal genes, containing only the big or the small intron, or with deletions at the borders of the introns have been constructed (F. MEYER, D. MARTI personal communication) and after introduction of the genes into L-cells (MANTEI et al. 1979) splicing can be studied.

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